

AUTOANTIBODIES TO A PULMONARY EPITHELIAL ANTIGEN IN
CRYPTOGENIC FIBROSING ALVEOLITIS.

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**Presented to the University of Edinburgh for the Degree
of Doctor of Philosophy
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Declaration

I declare that the studies presented in this thesis, which is submitted to the University of Edinburgh for the Degree of Doctor of Philosophy, are entirely my own work.

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transforming growth factor	153
tumour necrosis factor	

List of Abbreviations used in the Thesis

ABC	avidin-biotin complex
ARDS	Adult Respiratory Distress Syndrome
BAL	bronchoalveolar lavage
BCIP	5-bromo-4-chloro 3 indolyl phosphate
BOOP	Bronchiolitis Obliterans Organising Pneumonia
CFA	Cryptogenic Fibrosing Alveolitis
EAA	Extrinsic Allergic Alveolitis
ELISA	enzyme linked immunosorbance assay
FCS	foetal calf serum
ICAM	intercellular adhesion molecule
IGF	insulin like growth factor
IL	interleukin
ILD	interstitial lung disease
INF	interferon
kDa	kilo Daltons
KGF	keratinocyte growth factor
MCP	macrophage chemoattractant protein
MIP	macrophage inflammatory protein
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide
NBT	nitro blue tetrazolium
NP40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline, pH 7.2
SDS	sodium laural sulphate
TBS	tris buffered saline, pH 7.2
TGF	transforming growth factor
TNF	tumour necrosis factor

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Abstract

Cryptogenic fibrosing alveolitis (CFA) is a chronic inflammatory disease of the pulmonary interstitium which results in progressive scarring of the lung with loss of function and eventual death. The aetiology is unknown but the condition is believed to have an immunological pathogenesis with a persisting immunological reaction to as yet unidentified endogenous or exogenous antigen.

Studies presented in this thesis have shown for the first time that patients with CFA have a high incidence (95%) of circulating IgG autoantibodies to an antigen present in lung tissue. The antigen appears to be endogenous, being detectable in CFA, sarcoid and control lung tissue. Experiments conducted with IgG purified from patient plasma suggested that the antigen was associated with alveolar epithelial lining cells.

An antiserum to this antigen raised in a rabbit was shown to recognise the same antigen as the patient autoantibody using a series of absorption, immunoprecipitation and antigen capture experiments. Immunohistochemistry revealed that this antigen was associated with alveolar epithelial lining cells and appeared to be lung specific. Experiments using the rabbit antiserum and CFA patient plasma revealed that the same antigen was expressed in an alveolar epithelial cell line (A549). Studies conducted with these cells demonstrated that the antigen was principally intracellular and appeared associated with the microsomal compartment of the cell although its nature remains unknown. Despite this apparent intracellular location the antigen was detected in bronchoalveolar lavage samples from some patients with CFA. *In vitro* experiments culturing the A549 cells in the presence of the immune rabbit sera revealed an inhibitory effect on cell number suggesting some biological activity for the anti-sera.

The data presented in this thesis confirms that a humoral immune response is occurring in the lungs of patients with CFA to an alveolar epithelial cell antigen. The presence of this autoantibody raises the possibility of developing a serological test for the diagnosis and/or monitoring of disease activity in CFA.

The alveolar structure of the alveolar wall (reviewed Weibel 1981), which represents part of the pulmonary interstitium (see below), is composed of a small quantity of connective tissue, principally collagen and elastin which can be identified on the side of the capillary away from the epithelial surface. Associated with these fibres are a few mesenchymal cells with myofibroblastic features, occasional tissue macrophages and mast cells. Unlike other species, such as the rat, no mucosal associated lymphoid tissue or lymphatics are observed in the normal human alveolated lung although occasional lymphocytes may be seen (Pabst 1992).

CHAPTER 1.

INTRODUCTION.

1.1 Anatomy.

The lung is a gas exchange organ with an anatomical structure which brings air and blood into close proximity in order to facilitate diffusion of oxygen and carbon dioxide across a thin respiratory membrane. Air is carried from the external environment via a series of airways of decreasing calibre which each ultimately lead to a functional unit of lung, referred to as the acinus, or acinar unit (Weibel 1989) where gas exchange occurs.

Each acinar unit is composed of alveoli (reviewed Williams *et al* 1989) which share a common terminal bronchiole and collectively they comprise the respiratory gas exchange area where movement of oxygen and carbon dioxide occurs between the atmosphere and the blood. The alveolar walls, or septa, are lined by a thin luminal epithelium of so called type I alveolar epithelial cells (reviewed Schneeberger 1991). These cells have a flattened attenuated appearance and are believed to cover around 90% of the alveolar surface. As the basement membrane underlying these cells is commonly fused with that of the subjacent capillary endothelial cells this creates a mean diffusion distance for gas exchange of around 0.2 μ m. A second type of alveolar epithelial cells (type II cells) are also recognised which have a rounded structure, are more metabolically active than type I cells and cover the remaining 10% of the alveolar surface (reviewed Mason and Williams 1991). These cells are recognised as important in the production of surfactant which coats the luminal surface reducing surface tension and preventing alveolar collapse. These cells are also believed contain the stem cell compartment for alveolar epithelium and are known to proliferate following epithelial injury.

The internal structure of the alveolar wall (reviewed Weibel 1991), which represents part of the pulmonary interstitium (see below), is composed of a small quantity of connective tissue, principally collagen and elastin which can be identified on the side of the capillary away from the epithelial surface. Associated with these fibres are a few mesenchymal cells with myofibroblastic features, occasional tissue macrophages and mast cells. Unlike other species, such as the rat, no mucosal associated lymphoid tissue or lymphatics are observed in the normal human alveolated lung although occasional lymphocytes may be seen (Pabst 1992).

The term 'pulmonary interstitium' has been accurately defined to include the peribronchial, perivascular, interacinar and subpleural connective tissue of the lung (von Hayek 1960). However, 'interstitium' is more commonly used to describe the small amounts of connective tissue and intercellular spaces that exist within the alveolar septa of the acinar unit. In this thesis the term 'interstitium' will (unless otherwise qualified) be used in this context as this is the widely used perception of the term.

The lung can thus be regarded as having different anatomical compartments (airways, alveolar spaces, vessels and interstitium) all of which may be involved in disease processes resulting in lung injury (Lamb 1990). The effects that disease processes, whether primary or secondary to the lung, have on pulmonary physiology relate not only to the type and severity of lung injury but also its distribution within the various anatomical compartments of the lung (Nunn 1987a, Murray 1992). Unlike other organs, such as the liver and kidney, where injury of functional units tends to have a uniform distribution within the organ, considerable variation in the severity and pattern of injury between different acinar units may occur within the lung (Dail and Hammer 1993). These variations are believed to result from the effects of gravity and the physiological differences in perfusion and ventilation that occur between the apex and base of the lung (Nunn 1987b). Thus patterns of lung disease are dictated by the nature of the injurious agent, route of entry, pattern of distribution in the lung and the anatomical compartment that is primarily affected.

1.2 Interstitial Lung Diseases.

The interstitial lung diseases (ILD) constitute a diverse group of poorly understood conditions (Table 1.1) having in common a disease process which predominantly involves the 'pulmonary interstitium' (as defined above) although secondary involvement of the bronchial, vascular or intraluminal compartments of the lung may occur (Fulmer and Crystal 1979, Dail and Hammer 1993). The majority of ILDs are characterised by the development of an inflammatory process in this compartment with a subsequent increase in fibro-connective tissue (Keogh and Crystal 1981, Crystal *et al* 1984). This has led to these ILDs being described as interstitial pneumonias or diffuse interstitial fibroses (Liebow and Carrington 1967).

The commonest, and most poorly understood form of ILD, is cryptogenic fibrosing alveolitis (CFA) (Grant 1989, Katzenstein and Askin 1990, Hammer 1993). This condition, which is characterised by the development of inflammation in the alveolar septa with subsequent fibrosis has a unique distribution within the lung. The disease predominantly affects the sub-pleural regions of the lower lobes and spares the more central portions of the lung even in advanced cases. At the microscopic level this tendency to involve the periphery is also evident within acinar units. The central portion of the acinus is characteristically spared until late in the disease process and the airways are rarely involved (Hammer 1993).

As discussed above the distribution of disease within the interstitium may be influenced by the type of insult and its subsequent distribution in the lung. Thus while ILDs remain poorly understood an appreciation of the anatomical distribution of the process may indicate possible aetiological or pathogenetic mechanisms. In extrinsic allergic alveolitis, for example, a focal inflammatory process involving the more proximal acinar unit and bronchioles is characteristic reflecting the known aetiology involving inhaled antigen (Hammer 1993). The unique distribution of the disease process observed in CFA, however, does not fit with any known pattern of injury and suggests a more complex aetiology and pathogenesis.

Many of the ILDs, including CFA, predominantly involve alveolar septa (Dail and Hammer 1993, Katzenstein and Askin 1990) and may not involve other areas of the interstitium as defined above. As a result the term 'interstitial disease' or 'diffuse interstitial fibrosis' has often become synonymous with alveolar septal disease and does not necessarily imply involvement of the entire pulmonary interstitial compartment.

Table 1.1

Recognised conditions giving rise to the clinical entity of diffuse interstitial fibrosis (adapted from Cotran 1989).

Diffuse Interstitial Lung Diseases.

1. Cryptogenic fibrosing alveolitis
2. Sarcoidosis
3. Extrinsic Allergic Alveolitis
4. Bronchiolitis Obliterans Organising Pneumonia
5. Pneumoconioses
6. Drug Reactions
7. Radiation Pneumonitis
8. Infection
9. Lymphangitis Carcinomatosa
10. Smoking
11. Systemic Inflammatory Processes
12. Goodpasture's Syndrome
13. Idiopathic Pulmonary Haemosiderosis
14. Eosinophilic Pneumonia
15. Histiocytosis X
16. Alveolar Proteinosis
17. Lymphocytic Interstitial Pneumonitis

1.3 Historical Aspects of Diffuse Interstitial Fibrosis.

The recognition of diffuse interstitial fibrosis as a disease process is usually credited to Hamman and Rich (1935, 1944) but was probably first described in the German literature in 1897 (Rindfleisch) and again in 1907 (Sandoz). Hamman and Rich reported 4 patients with a rapidly progressive pulmonary illness leading to death over a period of 20 days to 3 months. At autopsy the lungs were characterised by the presence of interstitial scarring associated with inflammatory cells, alveolar epithelial cell hyperplasia, necrosis of alveolar and bronchiolar epithelium, oedema and intraluminal fibrin with hyaline membrane formation and the absence of identifiable causative organisms. While these patients were considered to have an acute pulmonary condition only 2 died of respiratory failure, the remaining 2 patients developed, and died as a consequence of, right heart failure suggesting some chronicity to the condition.

Potter and Gerber (1948) reported the first case of diffuse pulmonary fibrosis with a history of greater than 6 months and this was quickly followed by others (Stursa 1949, Beams and Harnos 1949, Golden and Tullis 1949). By 1956 when Grant *et al* reviewed 39 published cases, including 3 from Edinburgh, it was concluded that an insidious onset of the condition was more common and that average survival was in the order of 2½ years. This was confirmed in 1964 when Livingstone *et al* presented clinical, radiological and pathological data on 45 such patients showing that the majority had a chronic rather than a rapidly fatal course. Since then it has been realised that diffuse scarring of the interstitium of the lung (from all causes) is more common than originally thought. The most recent data from the Office of Population Censuses and Surveys (OPCS 1991) suggests that it has a prevalence of 1:3,000 - 4,000 in the UK and accounts for around 3,000 deaths annually.

Over 100 different causes of diffuse interstitial lung disease have now been described which can be grouped into various aetiological categories (Table 1.2). Acute, rapidly progressive, forms of diffuse pulmonary fibrosis are recognised to be relatively rare but have been described following infection with atypical organisms (Spencer 1985), inhalation of gases, dusts and fumes (Weill and Turner-Warwick 1981), exposure to systemic poisons such as paraquat (Vale and Meredith 1981) and as the adult respiratory distress syndrome (ARDS) arising as a complication of major sepsis or trauma (Hasleton 1983, Bartels and Reale 1991). This acute pattern of

disease appears to be more characteristic of the condition originally described by Hamman and Rich and subsequent review of their material has confirmed this (Olsen *et al* 1990). It is however recognised that an acute Hamman-Rich type pattern of lung injury may superimpose itself, possibly as a terminal event, in patients with more chronic forms of diffuse interstitial fibrosis (Pratt *et al* 1979, Guest *et al* 1993). Thus, rather than there being a clear distinction between acute and chronic forms of diffuse interstitial fibrosis they may represent different spectra of common patterns of lung injury and repair.

More commonly, diffuse interstitial fibrosis is of unknown aetiology and has an insidious onset with progressive lung damage. The largest single group of patients with diffuse interstitial fibrosis falls into this category with an incidence of between 5 and 10 per 100,000 of the population in the USA (Hammer 1993) and an annual mortality of some 1,500 in the UK (Johnstone *et al* 1990, OPCS 1991). This group of patients with idiopathic interstitial pulmonary fibrosis have been labelled with a variety of terms (see below) including cryptogenic fibrosing alveolitis (CFA), idiopathic pulmonary fibrosis (IPF) and usual interstitial pneumonitis (UIP).

Following the recognition that the majority of patients with diffuse pulmonary fibrosis were of unknown aetiology and showed similar clinical and pathological features attempts were made to define the condition in further detail. Liebow and Carrington (1967) attempted to describe the condition in greater pathological detail and divided cases on the basis of their histological appearances into 5 categories.

1. Usual Interstitial Pneumonitis (UIP) was described as showing lesions with a spectrum of appearances ranging from areas of acute alveolar damage with fibrin leak into the alveolar spaces reminiscent of that described by Hamman and Rich (1944) to areas of chronic inflammation with interstitial fibrosis.
2. Usual Interstitial Pneumonitis with Bronchiolitis Obliterans (BIP) where bronchiolar damage and bronchiolar luminal exudates were superimposed on the normal histological appearance of UIP.
3. Desquamative Interstitial Pneumonitis (DIP) (Liebow *et al* 1965) represented a relatively uniform pattern of the disease characterised by the accumulation of large numbers of mononuclear cell in the airspaces, a lack of necrosis and fibrinous exudate and minimal interstitial fibrosis. Initially the mononuclear cells were thought to represent desquamated alveolar

epithelial lining cells but subsequently were identified as alveolar macrophages (Fromm *et al* 1980).

4. Lymphocytic Interstitial Pneumonitis (LIP) (Carrington and Liebow 1966) was described as being characterised by a marked infiltrate of mature lymphocytes and plasma cells without nodular parenchymal masses of lymphocytes, germinal centre formation or local lymph node involvement.

5. Giant Cell Interstitial Pneumonitis (GIP) was described as a rare lesion characterised by the accumulation of bizarre giant cells in the alveolar airspaces.

No general agreement followed either on the terminology introduced by Liebow and Carrington or on the notion that these histologically defined patterns of disease were anything other than a spectrum of the same condition. Scadding (1964) criticised the use of both the term 'interstitial' which he thought anatomically imprecise (section 1.1) and 'pneumonia' or 'pneumonitis' which he felt should be retained for intraluminal inflammatory conditions. He suggested the term idiopathic or cryptogenic fibrosing alveolitis should be applied to the whole gamut of histological changes observed in these conditions. He later proposed that DIP and UIP might represent early and late stages of the same condition (Scadding and Hinson 1967) and suggested that the term 'mural fibrosing alveolitis' should be used for UIP and 'desquamative fibrosing alveolitis' for DIP in a further attempt to remove the terms 'interstitial' and 'pneumonitis' from the nomenclature.

Despite Scadding's attempts to remove these descriptive but imprecise terms they became accepted, particularly in the American medical literature, but not before the debate led to a profusion of names for the disease entity. In the UK cryptogenic fibrosing alveolitis (CFA) is favoured while in North America idiopathic pulmonary fibrosis (IPF) or usual interstitial pneumonitis (UIP) is more conventionally applied to this clinico-pathological entity of unknown aetiology.

Table 1.2 Clinical Features of Cryptogenic Fibrosing Alveolitis

Recognised aetiological categories of diffuse interstitial pulmonary fibrosis with examples (adapted from Hammer 1993). Ashin (1990).

<u>Aetiological Categories of Diffuse Interstitial Fibrosis.</u>	
Immunological Processes (eg. Extrinsic Allergic Alveolitis, Sarcoidosis)	
Viral Infections (eg EBV, Varicella zoster)	
Pneumoconioses (eg. asbestosis, silicosis, coal workers pneumoconiosis)	
Collagen Vascular Diseases (eg. rheumatoid arthritis, scleroderma, systemic lupus erythematosus)	
Adult Respiratory Distress Syndrome	
Drug Reactions (eg. bleomycin, nitrofurantoin, amioderone)	
Ionising Radiation	
Inhalation of Metals (eg. iron, mercury, cadmium)	
Idiopathic (sporadic and familial)	
Miscellaneous (Histiocytosis X, chronic cardiac failure, smoking)	

1.3.2 Microscopic Features

CFA is characterized histologically as an inflammatory and fibrosing process which shows wide variation in the degree of lung involvement from field to field within cases and between cases. This variability may be very marked with apparently normal acini or those with dense inflammatory infiltrates adjacent to end-stage honeycomb lung (Fig. 1.3). In terminal cases examined at autopsy, this variation may persist with much of the more central lung apparently spared and by degrees surrounded and replaced by areas of dense fibrosis. Even the areas of alveolar destruction and fibrosis have a heterogeneous appearance. Some are predominantly composed of mature collagenous tissue with a few interstitial cells whereas other areas show foci of proliferating mesenchymal cells associated with scanty extracellular matrix deposition.

1.4. Pathological Features of Cryptogenic Fibrosing Alveolitis.

The pathological features of CFA have been recently reviewed in detail by Hammer (1993) and Katzenstein and Askin (1990).

1.4.1 Macroscopic Features.

The gross pathological changes in the lungs of patients with CFA vary depending on the stage of the disease. In the early stages there may be few macroscopic abnormalities evident but in patients dying from advanced disease a characteristic appearance is observed at autopsy. The lungs are heavy (2-3 times normal) and stiff, remaining 'inflated' after removal from the chest. The pleura is often mildly thickened and diffusely nodular but the presence of fibrous adhesions to the chest wall are unusual.

On sectioning the most peripheral 2-3 cm of the lung shows the most striking abnormality while the proximal or central portion may be normal even in patients dying with end stage disease (Fig. 1.1). Characteristically the changes are more severe in the lower lobes than in the upper lobes. The peripheral area shows obliteration of the normal macroscopic acinar architecture with replacement by variable quantities of dense grey-white fibrous tissue. The scarring is often associated with the development of cystically dilated 'honeycomb' spaces which are the residual airways left in the tissue after obliteration of the remainder of the acinar unit (Fig 1.2). These honeycomb spaces may contain mucoid secretions or pus. Most patients with CFA die in cardio-respiratory failure and features of bronchopneumonia may frequently be observed at autopsy.

1.4.2 Microscopic Features.

CFA is characterised histologically as an inflammatory and fibrosing process which shows wide variation in the degree of lung involvement from field to field within cases and between cases. This variability may be very marked with apparently normal acini, or those with dense inflammatory infiltrates adjacent to end-stage honeycomb lung (Fig. 1.3). In terminal cases, examined at autopsy, this variation may persist with much of the more central lung apparently spared by the process. Even the areas of alveolar destruction and fibrosis have a heterogeneous appearance. Some are predominantly composed of mature collagenous tissue with a few mesenchymal cells whereas other areas show foci of proliferating mesenchymal cells associated with scanty extracellular matrix deposition.

The variability in appearance is such that Hammer (1993), in a study of 37 lobes from 20 patients with CFA, identified in each specimen areas which contained patterns of disease corresponding to several of the categories described by Liebow and Carrington (1967) (section 1.3).

Despite the heterogeneity in the overall architectural changes an underlying pattern may be observed. The inflammatory and fibroblastic process characteristically appears to have a distribution involving the more distal parts of the acinar unit with initial sparing of the central component and respiratory bronchioles. In areas of lung where the architecture is better preserved this can also be appreciated as a sub-pleural accentuation of the disease process. As the destruction progresses the remainder of the acinar unit becomes involved but the airway does not. The residual airways become surrounded by fibrous tissue and dilate giving rise to the honeycomb spaces which are characteristic of the end-stage pattern of fibrosis observed in CFA (Fig. 1.4).

The patchy chronic inflammatory infiltrate consists mainly of small lymphocytes, macrophages and plasma cells (Fig. 1.5) although increased numbers of mast cells may also be present. The lymphocytes form small focal aggregates although germinal centres are rare (Fig. 1.6). Neutrophils and eosinophils may be present and are often associated with evidence of fibrin leak into the adjacent airspaces suggesting focal acute lung damage (Fig. 1.7). There is a characteristic increase in the numbers of intraalveolar macrophages and variable numbers of lymphocytes, neutrophils and eosinophils may be identified in airspaces. Although occasional multinucleate macrophages may be identified, granuloma formation is not characteristic of CFA.

The fibrosis in CFA is usually well established by the time of diagnosis, but because of the heterogeneity in disease progression damaged acini containing inflammatory cells admixed with various amounts of collagen may be seen. In scattered foci within the tissue distinct aggregates or 'fibroblastic foci' can be observed composed of closely spaced fibroblasts with little collagen deposition (Fig 1.8). These are believed to be the result of incorporation of intraluminal fibroblastic buds that occur at sites of epithelial cell necrosis (Myers and Katzenstein 1988). These fibroblastic foci are characteristically associated with atypical hyperplastic type II cells (Fig 1.9) and appear to continue to proliferate after

incorporation into adjacent structures giving rise to a true interstitial fibroblastic process (McDonald 1991).

The alveolar lining cells in areas of lung damage show a characteristic pattern of proliferating cuboidal cells which have the ultrastructural appearances of type II alveolar epithelial lining cells (Kawanami *et al* 1982). These cells are often very prominent, may show frank cytological atypia and appear particularly associated with the fibroblastic foci described above. They may therefore represent evidence of epithelial regeneration at sites of injury. Focal bronchial and squamous metaplasia may also be observed, more commonly in areas of end-stage fibrosis.

Other changes in the mesenchymal cell population may also be present to various degrees. Proliferation of interstitial cells with a smooth muscle morphology may also be apparent. The pleura is usually thickened but rarely appears inflamed. There may be evidence of gross remodeling of the pulmonary vasculature with medial hypertrophy and eccentric intimal thickening. Vessels may show evidence of re-canalisation suggesting previous occlusion by thrombus possibly due to local inflammatory activity.

Liebow and Carrington (1967) in their attempts to classify histological variants of CFA recognised a group of patients with apparent bronchiolar damage (BIP) superimposed on the more normal pattern of airway sparing (see section 1.3). Despite this, small airway damage is not recognised as being characteristic of CFA (Katzenstein and Askin 1990, Hammer 1993). In Edinburgh our experience has confirmed that occasional biopsies showing features of CFA may show in addition focal evidence of a more proximal disease process. This is characterised by the presence of a mild bronchiolitis with submucosal thickening and organising alveolar exudates in the proximal part of the acinus. The explanation for such changes is unclear. It is however recognised that patients with CFA may present to chest physicians with symptoms relating to an upper respiratory tract infection (Herbert *et al* 1962, Liebow and Carrington 1967). Biopsy at this time might, therefore, result in the superimposition of extra pathological features on those of the underlying interstitial lung disease. Thus while airway damage may not be a characteristic of CFA it may on occasion be seen as an associated feature.

Figure 1.1

Photograph illustrating the macroscopic appearance of a para-sagittal slice of lung from a patient who died of CFA. Extensive fibrosis with honeycomb change can be seen (H) especially at the apex of the lower lobe. The fibrosis shows a marked sub-pleural distribution which can be best appreciated anteriorly in the upper lobe which is less severely affected (arrow) by the disease process. Despite the fact that this patient died as a consequence of CFA much of the central part of the lung is spared and appears relatively uninvolved (N).

Figure 1.2

Photograph illustrating the macroscopic appearance of honeycomb lung as observed in areas of end stage pulmonary fibrosis from a patient with CFA. Large dilated spaces with fibrous walls are present. These represent the remnants of bronchioles in the lung and are frequently filled with mucus and pus at autopsy where death from respiratory failure is common.

Figure 1.1

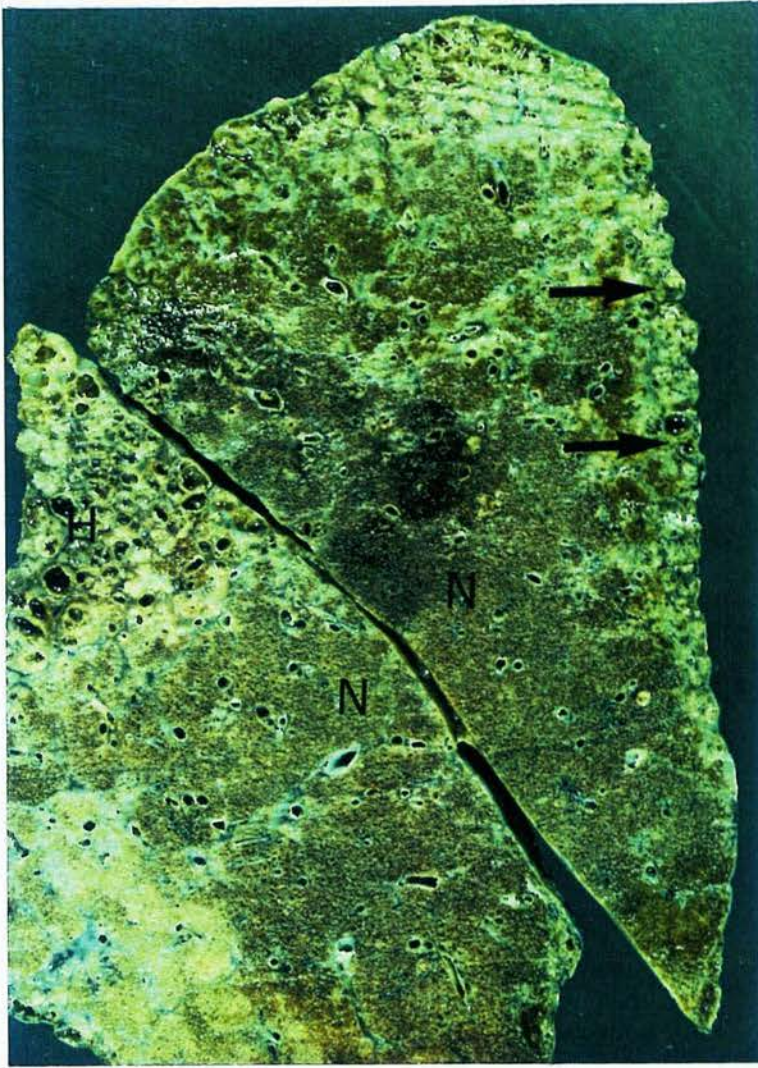


Figure 1.2

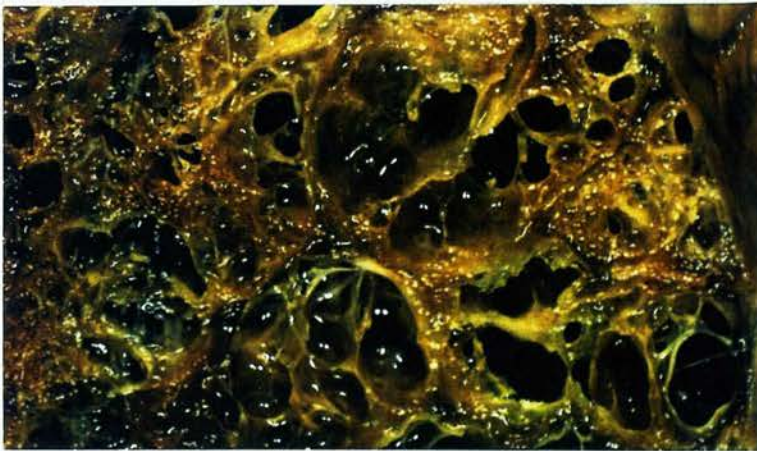


Figure 1.3

Photomicrograph from an open lung biopsy from a patient with CFA illustrating the variable and patchy nature of the disease process. An oedematous septum is present in the centre of the picture (S). The adjacent lung parenchyma to the left appears essentially normal (N) with no evidence of significant inflammation or interstitial scarring. The lung parenchyma to the right of the septum, however, shows extensive fibrosis with the formation of honeycomb spaces (H) and a focal chronic inflammatory infiltrate. (Haematoxylin and eosin; original magnification X40).

Figure 1.4

Photomicrograph from an open lung biopsy from a patient with CFA illustrating the histological appearances of end stage pulmonary fibrosis in CFA with honeycomb space formation (H). The honeycomb spaces are shown to contain inspissated mucus. While occasional chronic inflammatory cells are noted in the fibrous tissue no significant residual inflammatory activity is evident in the tissue. (Haematoxylin and eosin; original magnification X100)

Figure 1.3

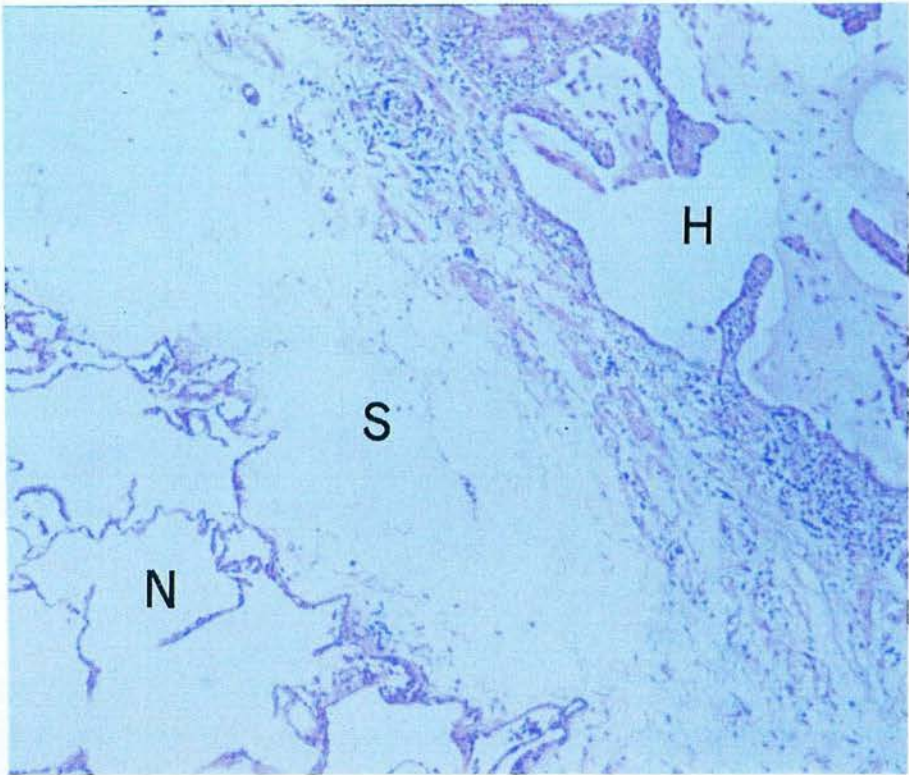


Figure 1.4

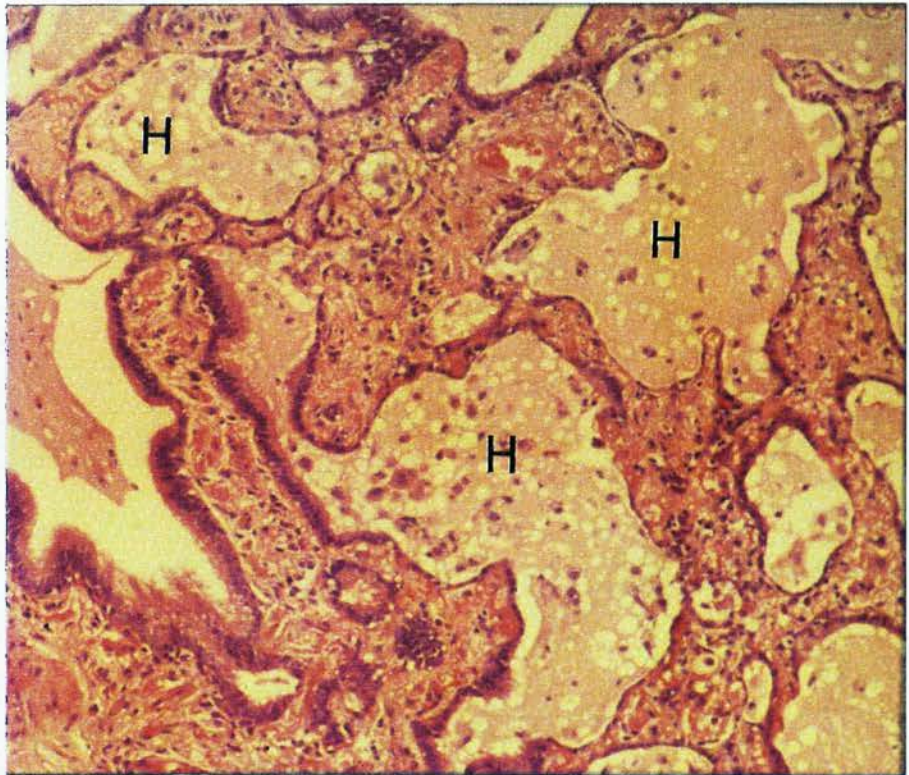


Figure 1.5

Photomicrograph from an open lung biopsy from a patient with CFA illustrating the presence of a diffuse chronic inflammatory infiltrate composed of lymphocytes (L), macrophages (M) and plasma cells (P). The intensity of the infiltrate is recognised to show considerable variability both within and between cases and has been suggested as a possible marker of response to immunosuppressive therapy (see section 1.7). Immunohistochemical studies have indicated that the majority of infiltrating lymphocytes are T cells (Campbell *et al* 1985, Kradin *et al* 1986) with approximately equal numbers of CD4 and CD8 positive cells. (Haematoxylin and eosin; original magnification X200)

Figure 1.6

Photomicrograph from an open lung biopsy from a patient with CFA illustrating the presence of a discrete aggregate of lymphocytes. Immunohistochemical studies have shown these to be principally composed of B lymphocytes associated with follicular dendritic cells. The immunohistochemical features of these aggregates have been suggested to be reminiscent of mucosal associated lymphoid tissue (MALT) that may be observed in other tissues (Wallace *et al* 1994) and may reflect local humoral immune activity. (Haematoxylin and eosin; original magnification X200)

Figure 1.5

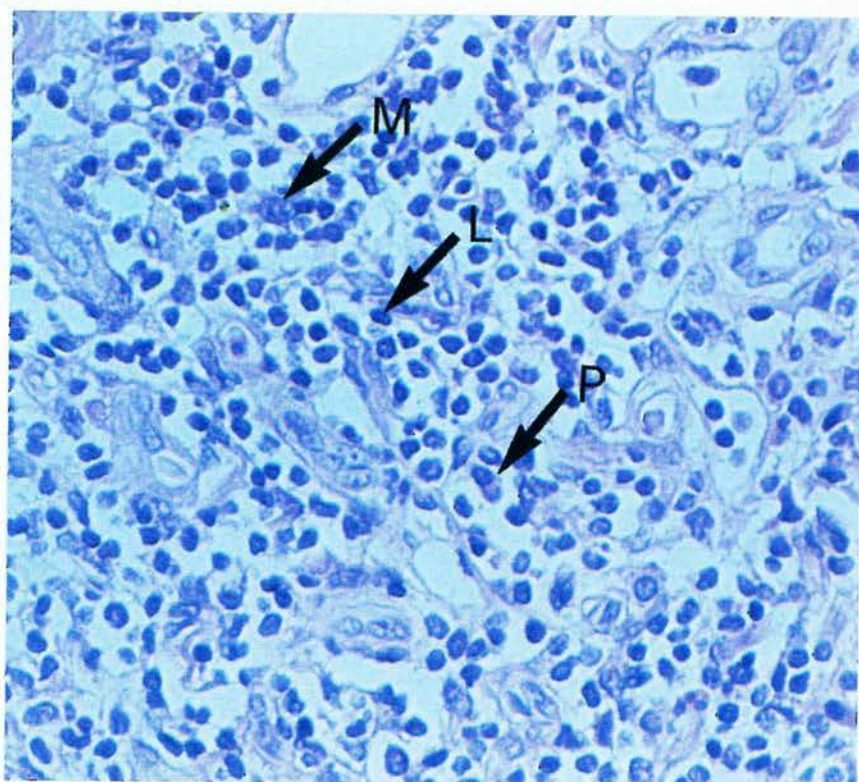


Figure 1.6

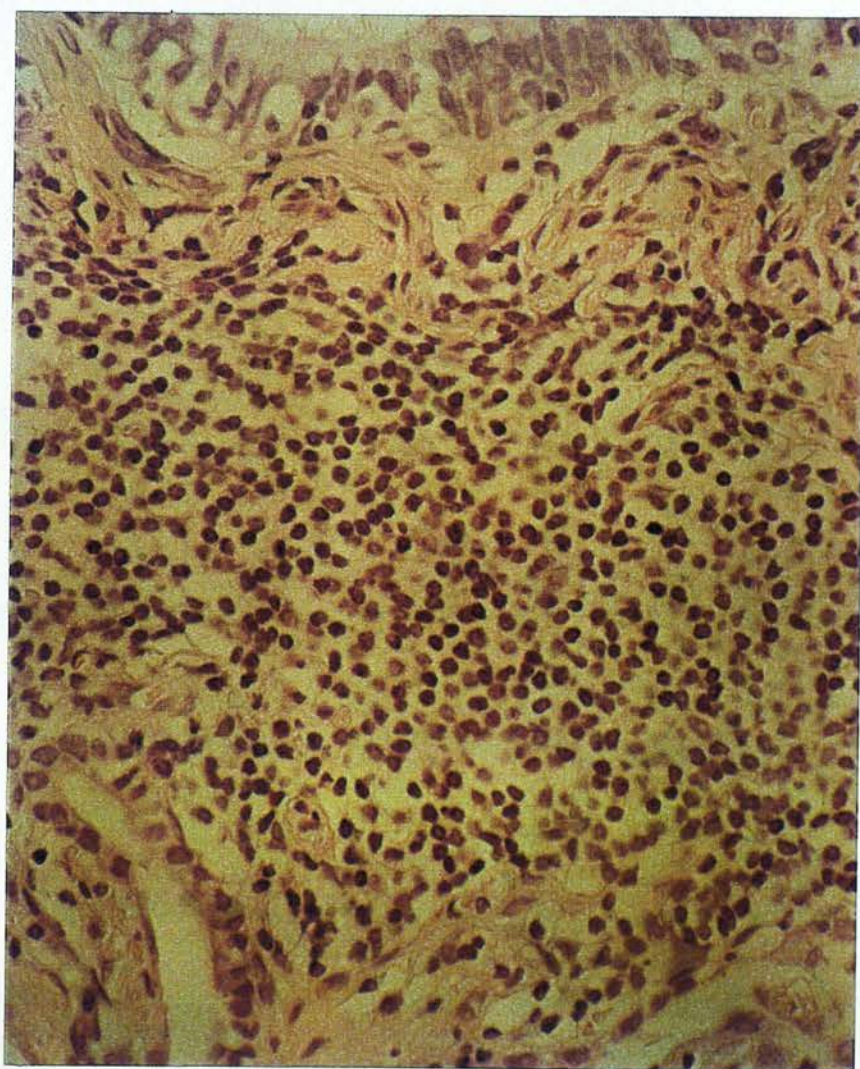


Figure 1.7

Photomicrograph from an open lung biopsy illustrating the presence of macrophages (M), lymphocytes (L), neutrophils (N) and eosinophils (Eo) in the alveolar airspaces of a patient with CFA. Hyperplastic type II alveolar epithelial cells (E) are also shown. CFA is characteristically associated with an increase in alveolar macrophage number which in some cases can become very marked. The presence of other intraluminal inflammatory cells is more variable and may be associated with the presence of a fibrin rich protein exudate. (Haematoxylin and eosin; original magnification X400)

Figure 1.7

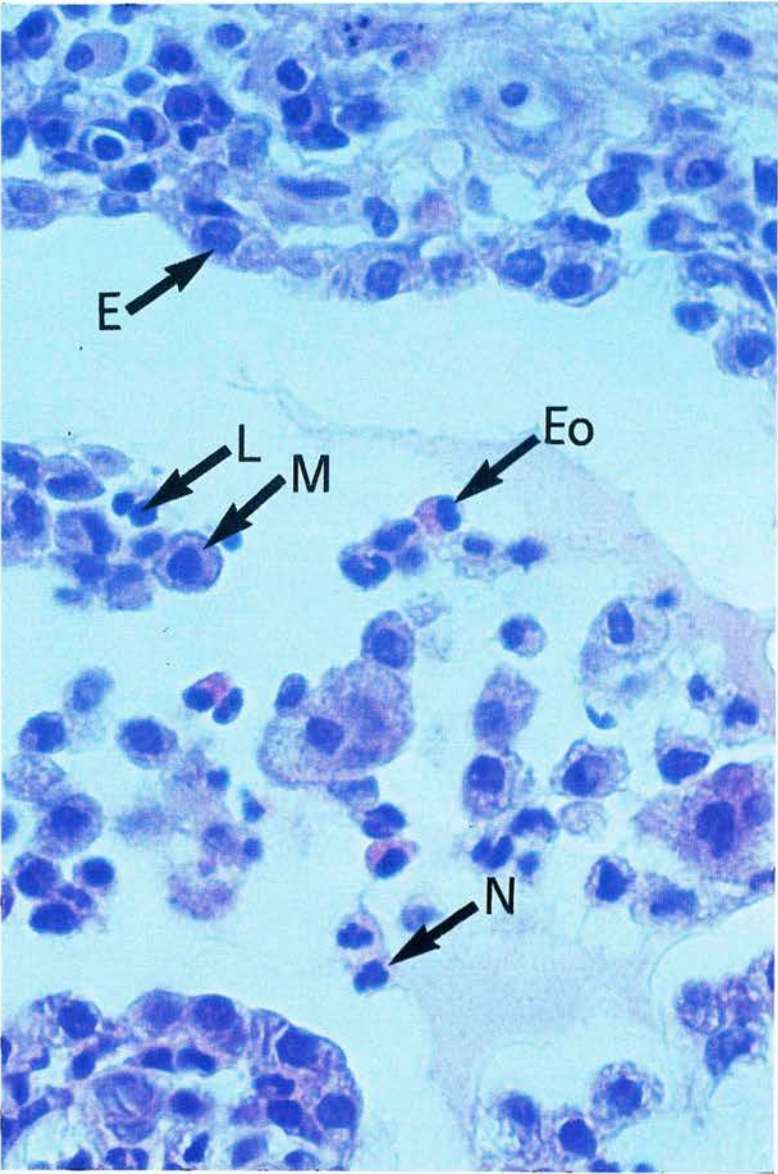


Figure 1.8

Photomicrograph from an open lung biopsy illustrating the presence of a 'fibroblastic focus' (F) which is being re-epithelialised by hyperplastic type II alveolar epithelial cells (E). The adjacent lung shows a moderate chronic inflammatory infiltrate. The 'fibroblastic foci' are believed to arise as a result of local epithelial cell injury with subsequent formation of a protein exudate and fibroblast migration to form a fibroblastic bud. The bud then appears to contract and becomes re-epithelialised so that the 'fibroblastic focus' is interstitial rather than intraluminal in position. (Haematoxylin and eosin; original magnification X40)

Figure 1.9

Photomicrograph from an open lung biopsy illustrating the appearance of hyperplastic type II epithelial cells in a lung biopsy from a patient with CFA. The cells show a considerable degree of nuclear variability although the reasons for this are unclear. In some cases occasional multinucleated cells or cells with nuclear inclusions may be seen. Atypical hyperplastic type II cells are frequently found associated with areas of active fibroplasia and may play a role in regulating fibroblast function (see section 1.10.6). (Haematoxylin and eosin; original magnification X200)

Figure 1.8

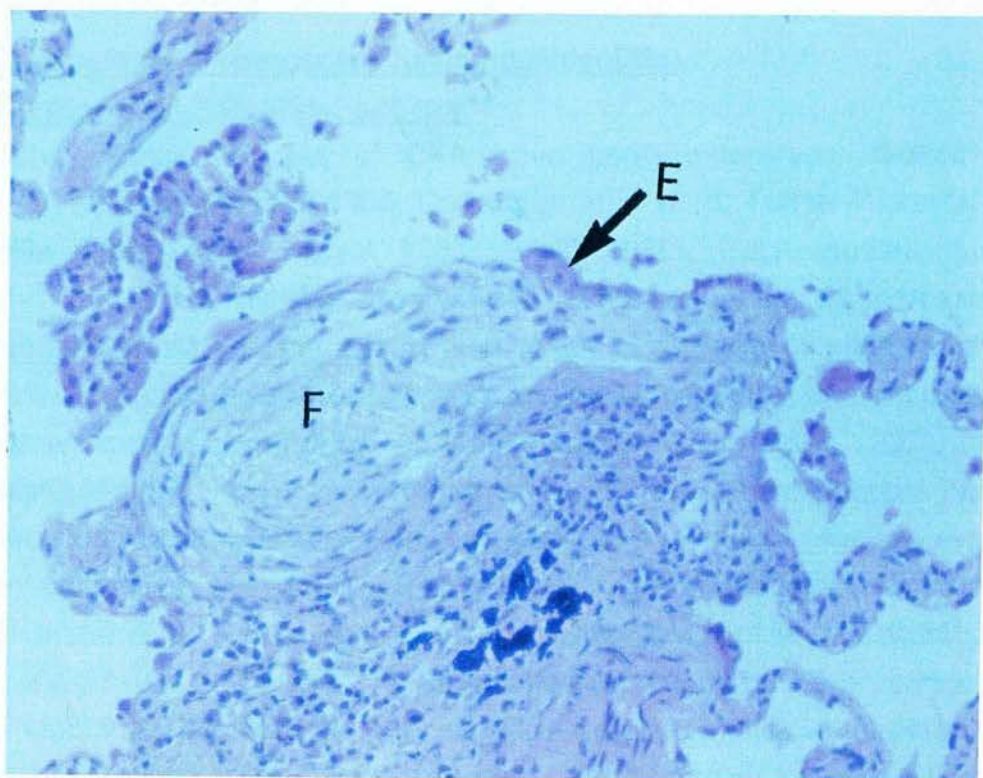
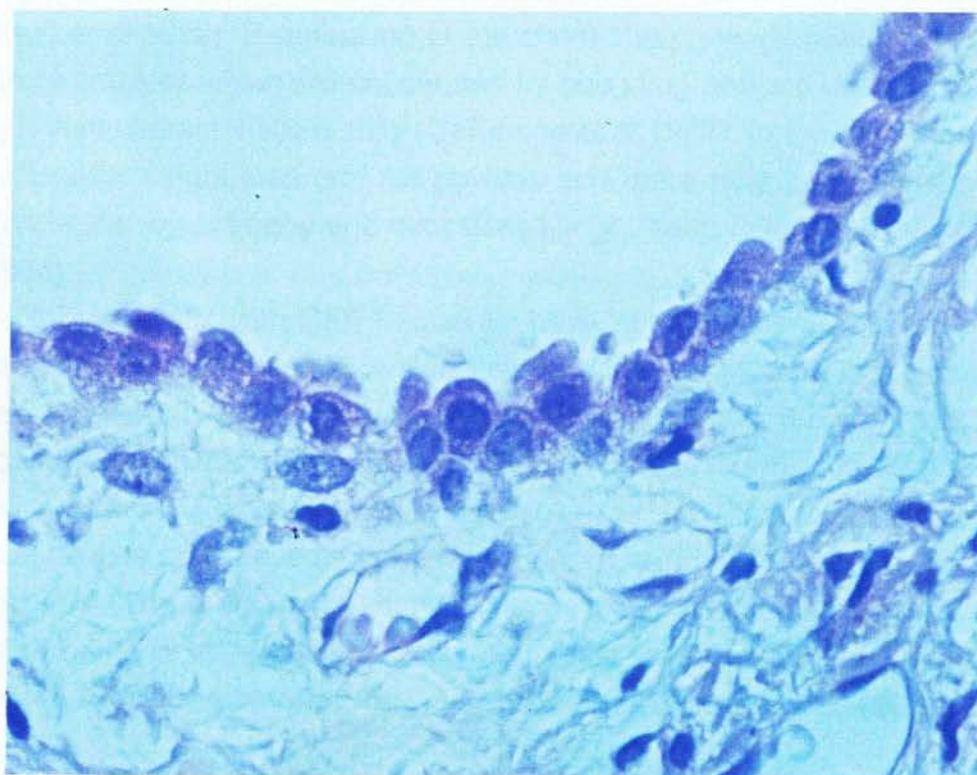


Figure 1.9



1.5. Diagnosis of Cryptogenic Fibrosing Alveolitis.

1.5.1 Clinical and Laboratory Features.

The clinical features of CFA have been extensively described (Scadding 1960, Stack *et al* 1965, Carrington *et al* 1978, Turner-Warwick *et al* 1980a, Hance and Crystal 1983, Grant 1989). Such studies have indicated that most patients present as a consequence of increasing shortness of breath initially on exertion. A smaller group of patients are, however, recognised to present with an acute episode of respiratory symptoms, resembling a flu like illness (Liebow and Carrington 1967), and on investigation are found to have pulmonary fibrosis. A few patients have also been described where presentation has been an acute terminal event with no antecedent symptoms but at autopsy have established pulmonary fibrosis (Pratt *et al* 1979). Patients may also complain of a persistent dry cough, weight loss and fatigue, repeated chest infections or the presence of finger clubbing. Occasional patients are asymptomatic and detected because of an abnormal chest x-ray performed for other reasons (Marks 1967, Scadding 1974).

The results of physical examination vary depending on the stage of disease progression. There may be tachypnoea on exertion or at rest with associated tachycardia (Rubin and Lubliner 1957, Marks 1967). Finger clubbing has been described in over two-thirds of CFA patients (Marks 1967, DeRemee *et al* 1972). Examination of the chest often reveals bilateral basal inspiratory crackles which are not cleared by coughing and are described as having a very characteristic quality (DeRemee *et al* 1972). In the later stages of the disease weight loss may be obvious and there may be evidence of right ventricular hypertrophy and occasionally right heart failure (Livingstone *et al* 1964).

While patients with CFA frequently have abnormalities in laboratory investigations they are usually of little help in establishing the diagnosis as they are non-specific in nature (reviewed Hance and Crystal 1983). Hypergammaglobulinaemia is common with an increase in either the α_2 or γ -globulin fraction (Crystal *et al* 1976). Quantitation of specific immunoglobulins often reveals polyclonal increases in one or more classes. IgG and IgM (Turner-Warwick and Doniach 1965, Turner-Warwick 1978, Crystal *et al* 1976) are frequently elevated while IgA has a more variable pattern and may be decreased in some patients (Turner-Warwick 1978). Up

to 50% of patients may show evidence of immune dysregulation with the development of non-specific immunological reactions which are usually associated with the collagen vascular diseases (section 1.9) (reviewed Hance and Crystal 1983). Several reports have also suggested that serum lactate dehydrogenase may be preferentially elevated in CFA compared with other forms of ILD possibly reflecting alveolar macrophage activation (DeRemee 1968, Matusiewicz *et al* 1993).

Patients with CFA show a classical restrictive pulmonary defect with reductions in total lung capacity, vital capacity, functional residual capacity and residual volume (Crystal *et al* 1976). The diffusing capacity as assessed by the carbon monoxide transfer factor is also usually reduced (Benson and Hughes 1972). Arterial blood gasses typically show hypoxaemia with a respiratory alkalosis which is typically exacerbated by exercise where a precipitous fall in the pAO_2 and oxygen saturation may be provoked (Crystal *et al* 1976, Hance and Crystal 1983). This has been suggested as a useful indicator of physiological impairment in patients with suspected CFA at an early stage in the disease where lung volumes and diffusing capacity may be normal (Scadding 1974, Carrington *et al* 1978).

1.5.2 Radiology.

Standard chest radiography in patients with CFA characteristically shows a fine reticulo-nodular pattern of shadowing particularly pronounced in the lower zones and towards the periphery (Livingstone *et al* 1964, Crystal *et al* 1976, Gaensler and Carrington 1980). In latter stages of the disease as fibrotic honeycomb changes develop (see section 1.4) these may also become apparent (Genereux 1975). The appearances are, however, recognised as being non-specific and thus chest radiographs are generally regarded as having a very low sensitivity and specificity for the diagnosis of CFA (Scadding and Hinson 1967, Carrington *et al* 1978, Grenier *et al* 1994). This is further complicated by the observation that up to 10% of patients in the early stages of the disease may have a normal chest x-ray (Scadding and Hinson 1967, Scadding 1974, Rubin and Lubliner 1957, Liebow and Carrington 1965, Carrington *et al* 1978).

The introduction of high resolution computerised tomographic scanning (HRCT scanning) over the last 5 years has increased the role of radiology in the diagnosis of CFA and other interstitial lung diseases (Strickland 1989, Muller and Miller 1990). Several studies have indicated

that HRCT can identify disease before abnormality is detectable on the chest x-ray (Muller 1991) and that it has a higher diagnostic accuracy (Mathieson *et al* 1989, Tung *et al* 1993, Grenier *et al* 1994). Tung *et al* (1993) reporting data from large tertiary referral centre with considerable experience of ILD concluded that CFA could be differentiated from other causes of diffuse pulmonary fibrosis using HRCT. They claimed a diagnostic accuracy of 88% for CFA and only an 11% false negative rate. Whether such results can be obtained in less specialised units is unclear and it is interesting to note that they claimed a high diagnostic accuracy for CFA using chest x-ray alone (76%) in contrast to other groups conducting similar studies (58%, Grenier *et al* 1994).

Some authors have, however, admitted that while the classical case of CFA is diagnosable easily by HRCT problems may exist in differentiating it from other diseases where there is extensive fibrosis (Strickland 1989). Thus while HRCT may have a role in the diagnosis of CFA, particularly in large specialist centres, further investigation may still be required to confirm the diagnosis.

1.5.3 Bronchoalveolar Lavage.

Bronchoalveolar lavage (BAL) was first described by Harris *et al* (1970) as a method of studying the cellular and protein content of the lung. The technique quickly became popular as a research tool to study changes in the cellular composition of the lungs in pulmonary diseases including CFA (Reynolds *et al* 1977, Cherniack *et al* 1990).

Studies to assess the diagnostic potential of BAL in CFA have suggested that an airspace lymphocytosis associated with granulocytes (neutrophils and eosinophils) is more characteristic of CFA than other forms of ILD (Reynolds *et al* 1977, Haslam *et al* 1980, Cherniack *et al* 1990). Despite this, a review of current practice with regard to the investigation of CFA found that in the UK only 17.5% of patients in which CFA was clinically suspected underwent lavage (Johnston *et al* 1993) compared with around 28% in the USA (Smith and Moser 1989). These statistics reflect the fact that BAL is seen as having a low diagnostic yield by chest physicians although studies by Drent *et al* (1993) examining data from 277 patients with either CFA, sarcoidosis or extrinsic allergic alveolitis using discriminate analysis of BAL and demographic data showed that 93% of the cases could be assigned to the correct diagnostic group.

1.5.4 Biopsy.

Definitive diagnosis of CFA currently requires histological examination of lung tissue (Wall *et al* 1981, Lamb 1990). The optimal method of obtaining such samples is by mini-thoracotomy (Wall *et al* 1981) or more recently thoracoscopy (Carnochan *et al* 1994). Such techniques while giving a high degree of diagnostic accuracy with a low complication rate (Wall *et al* 1981) do involve a surgical procedure and as such have attracted some controversy (Murray 1980).

As an alternative fibre-optic bronchoscopy provides a safe and easily performed technique with which small biopsies of the peripheral lung can be performed (Levin *et al* 1974). The ease with which the material can be obtained is, however, set against the small size of the biopsies and is thus subject to considerable sampling error. Nevertheless, transbronchial biopsies may be of use in diagnosing certain forms of ILD, particularly those with a more bronchocentric pattern such as sarcoidosis and extrinsic allergic alveolitis and excluding other causes of diffuse lung infiltration such as lymphangitis carcinomatosa (Wall *et al* 1981).

The technique is, however, of no value in obtaining a positive diagnosis in cases of CFA as this requires histological assessment of disease pattern (see section 1.4) which cannot be performed on small biopsies (Lamb 1990, Katzenstein and Askin 1990, Hammer 1993). Wall *et al* (1981) in a series of 53 patients with diffuse interstitial fibrosis found that a specific diagnosis was obtained with transbronchial biopsy in only 37% and that this was increased to over 90% if subsequent open lung biopsy was performed. Transbronchial biopsies from patients with CFA usually showed a degree of chronic interstitial inflammation with some fibrosis neither of which was specific.

Wall *et al* (1981) also reviewed the published series of diagnostic results obtained with various surgical lung biopsy techniques in unselected patients with diffuse interstitial disease. In 15 published series of 2,290 patients undergoing open lung biopsy a 94% diagnostic rate was obtained with 1.8% mortality and 7% complication rate. In contrast 1,340 needle core biopsies and drill biopsies showed a significantly lower diagnostic yield (63 and 72% respectively) and a higher complication rate (over 40%). They concluded that in patients with diffuse interstitial lung disease a non-specific

or normal transbronchial biopsy should be followed by surgical biopsy and that open procedures were safe and gave the highest diagnostic yield.

A recent survey of the management of CFA patients in the UK revealed that only around one third of 200 CFA patients diagnosed on clinical grounds underwent transbronchial biopsy (Johnston *et al* 1993, 1994a). Despite the evidence that such biopsies cannot give a histological diagnosis, 40% of the biopsies were regarded by the physician or pathologist as being diagnostic of CFA and only 7.5% of patients were referred for open biopsy. This contrasts with clinical practice in the USA where 75% of patients undergo transbronchial biopsy and 42% open biopsy (Smith and Moser 1989).

Thus while transbronchial biopsy may have role in excluding other conditions a formal open biopsy is required to provide histological proof of the diagnosis of CFA. The low rate of obtaining a definitive diagnosis in the UK makes epidemiological and clinical studies difficult to assess (Costabel 1993). Experience in Edinburgh over the last 3 years, where most patients with ILD have been referred for thoracoscopic lung biopsy, has suggested that around 10-15% of patients with suspected CFA will have either a pattern of non-specific scarring not suggestive of CFA or a completely different diagnosis (personal observation). Such experience suggests that research on patients with CFA, where the diagnosis has not been confirmed histologically, must be interpreted with caution as a significant number of the subjects may not have the condition.

1.6. Epidemiology of Cryptogenic Fibrosing Alveolitis.

CFA has a quoted incidence of 5 - 10 per 100,000 in the UK and North America (Hammer 1990, OPCS 1990, Johnston *et al* 1990) and recently it has been suggested that this figure may be rising (Johnston *et al* 1990). As has been discussed above, however, the diagnosis of CFA is difficult in life and is rarely confirmed histologically. Thus estimates of incidence based on diagnostic coding data and death certification rates are potentially liable to substantial error and care has to be taken before asserting that the condition is becoming more common.

CFA has been described in all age groups from childhood (Diamond 1958, Hewitt *et al* 1977) to patients over 100 years old (Johnston *et al* 1994b). The median age of onset of symptoms appears to be in the 6th or 7th decade with no apparent sex difference (Livingstone *et al* 1964, Crystal

et al 1976, Turner-Warwick *et al* 1980a, Johnston *et al* 1994a). Most cases appear to be sporadic although the occurrence of disease in twins (Peabody *et al* 1950, Javaheri *et al* 1980) and family clusters, some of which have other associated diseases, is well recognised (Ellis 1965, Bonanni *et al* 1965, Davies and Tuddenham 1976, Demedts *et al* 1976).

1.7 Staging of Patients with Cryptogenic Fibrosing Alveolitis.

Prior to embarking on treatment programmes for patients with CFA it is necessary to establish a firm baseline evaluation of the patients disease. The purpose of this evaluation is to establish the probability of a successful response to therapy and the current degree of lung impairment so that the progression may be monitored.

1.7.1 Assessment of the Alveolitis.

Several groups have found that a prediction of response to therapy may be made by assessing open lung biopsy specimens. Patients with cellular biopsies containing a prominent inflammatory infiltrate appear to respond more favourably than those showing predominantly fibrosis (Scadding and Hinson 1967, Turner-Warwick *et al* 1980b, Carrington *et al* 1978) suggesting that the presence of an active alveolitis may predict a beneficial response to therapy. On this basis several scoring systems have been described to stage the disease in a more reproducible fashion (Scadding and Hinson 1967, Gaensler and Carrington 1980, Ashcroft *et al* 1988, Cherniack *et al* 1991a) but it is unclear whether this is more accurate than subjective pathological assessment of the biopsies cellularity. The use of biopsies to predict response is, however, subject to the limitations of sampling error. Staging of the disease can only be carried out on the material supplied which may not reflect activity in the lung as a whole and in addition cannot be easily repeated.

In order to try and develop a reproducible method of assessing the activity of the alveolitis various groups have examined the relationship between BAL differential counts and response to therapy (Haslam *et al* 1980, Rudd *et al* 1981). While the total cellularity of the lavage appears more related to smoking habit than disease (Schwartz *et al* 1991) the presence of increased numbers of neutrophils or eosinophils appeared to be associated with a poorer response. Turner-Warwick *et al* (1987) showed that an objective response to steroid was associated with a fall in the granulocyte

content of subsequent BAL samples in contrast to those who did not respond suggesting that this might provide a mechanism for monitoring disease activity. Interestingly they also noted a subgroup of patients who had elevated BAL granulocytes, who did not respond to steroids but who appeared to have comparatively stable disease. Sampling error may, therefore, also be a problem with BAL as the disease process is heterogeneous and the percentage of neutrophils in the lavage may be distorted if pus filled honeycomb spaces are sampled.

Non-invasive techniques have also been investigated in order to assess the activity of the alveolitis. Lee *et al* (1992) and Wells *et al* (1993a) have correlated the presence of alveolitis in biopsy specimens with a 'ground glass' appearance on HRCT and shown that this is predictive of response to therapy. The same group (Wells *et al* 1993b) and others (Line *et al* 1978) have also used pulmonary isotope scanning to predict activity which they claim can offer similar information on likely response to therapy but others have found this to be poorly reproducible (King TE, Denver, Colorado. personal communication).

1.7.2 Assessment of Disease Progression.

While the presence of an alveolitis appears to be important in assessing the likelihood of a response to therapy patient morbidity and ultimate mortality are dictated by the fibrotic process. Disease activity can therefore also be examined from the perspective of fibroplasia and the deposition of extracellular matrix components. Routine chest radiographs are recognised to be insensitive for this purpose (Turner-Warwick *et al* 1980a) although HRCT may be better (Wells *et al* 1993a) as it appears to be able to differentiate fibrotic lung from inflamed and normal areas. Serial pulmonary function testing, particularly reductions in lung volumes and carbon monoxide transfer factor are recognised to reflect increasing fibrosis (Benson and Hughes 1972, Crystal *et al* 1976) but may not be very reproducible in breathless patients. Recently interest has focused on exercise induced desaturation as a more reproducible method of assessing disease progression (Crystal *et al* 1976, Ralph *et al* 1994).

Studies of open lung biopsy tissue has shown that areas of immature scar tissue are characterised by the deposition of type III collagen which appears to be replaced by type I collagen as maturation occurs (Bateman *et al* 1981). Serum and BAL type III procollagen peptides have thus been

measured as a marker of type III collagen synthesis (Low *et al* 1992) and have been found to correlate with non-specific markers of inflammatory activity such as C reactive protein and changes in alveolar oxygen gradients induced by exercise testing. Other groups have found, however, that BAL levels of these peptides show considerable heterogeneity, possibly due to variations in phagocytic clearance in the lung, making their use in clinical assessment difficult (Harrison *et al* 1993). A recent NHLBI Workshop (Cherniack *et al* 1991b) reviewed the problems of diagnosis, disease monitoring and therapy in CFA and concluded that little progress could be made in improving the clinical outlook of such patients until further progress had been made in developing reproducible tests that would allow accurate monitoring of disease activity and response to therapy.

1.8. Prognosis and Therapy of Patients with Cryptogenic Fibrosing Alveolitis.

Clinical studies of patients with CFA have indicated that the median time from diagnosis to death is 3 to 5 years (Scadding 1960, Scadding and Hinson 1967, Stack *et al* 1972, Turner-Warwick *et al* 1980b, Carrington *et al* 1978) although those with associated connective tissue disorders appear to have a less rapidly progressive disease (Agustini *et al* 1992). Most patients die from cardio-respiratory failure but an increased incidence of death from ischaemic heart disease is also recognised (Stack *et al* 1972) as is an increased risk of pulmonary carcinoma (Stack *et al* 1972: 7%, Turner-Warwick *et al* 1980c: 9.8%, representing a relative excess risk of 14.1) which is not accounted for by age, sex or smoking history.

Traditionally corticosteroids have been used in the treatment of CFA (Crystal *et al* 1976, Meir-Sydow *et al* 1980, Turner-Warwick *et al* 1980b). It is, however, recognised that the majority of patients fail to respond to show an objective response and those that do (10-20%) appear to show only a moderate increase in survival (Stack *et al* 1972, Turner-Warwick *et al* 1980b, Meir-Sydow *et al* 1980) with most dying within 10 years. In the belief that the disease is the result of an immunologically mediated, possibly autoimmune, process patients have been treated with a wide range of immunosuppressive agents including azathioprine (Wesse *et al* 1975, Winterbauer *et al* 1978), cyclophosphamide (Johnson *et al* 1989, Hunninghake *et al* 1980), cyclosporin (Moolman *et al* 1991) and penicillamine (Meir-Sydow *et al* 1979) in an attempt to improve the prognosis. While benefits in individual patients

and in small trials have been reported no definitive evidence of a role for any of these agents has been established (Cherniack *et al* 1991b). A recent survey of therapeutic approaches to CFA in the UK found that 34% of patients were not treated, 55% received a trial of steroid therapy and only 10% were given other immunosuppressive agents (Johnstone *et al* 1993). Similar findings have been reported from the USA (Smith and Moser 1989).

Clinical studies have indicated that younger, female patients with short histories and well preserved lung function at the time of diagnosis are more likely to show objective responses to therapy (Turner-Warwick *et al* 1980a). More recently Schwartz *et al* (1994) confirmed that male gender, the severity of symptoms and a smoking history are adverse prognostic factors. What is unclear is whether these studies detect a subgroup of patients with a particular pattern of disease which is more responsive to therapy, or patients who simply present at an earlier stage of the same disease. The latter is certainly suggested by the correlation of survival time with better preserved lung function at the time of diagnosis. This raises the possibility that the improved survival may represent a 'lead time' effect rather than a genuine sub-group of patients with a disease pattern more susceptible to therapy. The two groups of patients who do appear to have a genuine better prognosis are those who have a desquamative pattern of disease (Carrington *et al* 1978) and those with an associated collagen vascular disease (Agustini *et al* 1992).

Currently the most promising therapeutic option, at least for younger patients, would appear to be pulmonary transplantation (Lancet Editorial 1992) although if, as is believed, the disease has an autoimmune component then recurrence of the disease might be predicted in the transplant. While this has not been reported in CFA disease recurrence in transplanted lungs of patients with other forms of ILD has been described (Johnson *et al* 1993, Frost *et al* 1993).

1.9. Aetiology of Cryptogenic Fibrosing Alveolitis.

Although the aetiology of CFA is unknown there is a recognised association with the collagen vascular diseases, especially rheumatoid arthritis, systemic lupus erythematosus and scleroderma (Huninghake and Fauci 1979). This group of patients in which CFA is associated with a systemic disorder may account for approximately 10% of all cases but the lung involvement is histologically indistinguishable from the so called " lone

CFA" (Lie 1993). A further 40-50% of patients show variable evidence of systemic dysregulation of their immune system with hypergammaglobulinaemia (Crystal *et al* 1976), circulating immune complexes (Dreisen *et al* 1978, Turner-Warwick 1978, Pratt *et al* 1979, Haslam *et al* 1979 and 1982, Crystal *et al* 1984) and the development of non-organ specific autoantibodies (Turner-Warwick and Doniach 1965, Turner-Warwick *et al* 1980a, Nagaya *et al* 1969 and 1972, DeRemee *et al* 1972, Haslam *et al* 1975) but with no clinical evidence of a systemic collagen vascular disease. CFA has also been reported associated with various types of immunologically mediated liver disease (Lynne-Davies and Sproule 1967, Turner-Warwick 1968) and inflammatory bowel disease (Wallaert *et al* 1985, Mathan *et al* 1989).

Genetic factors have been suggested as playing a role in the aetiology of CFA following the recognition that family clusters may occur and reports of concurrent disease in monozygotic twins (see section 1.6). No conclusive evidence of an association with particular HLA haplotypes has been found, although one group did find a possible association with HLA B8 in patients with CFA presenting under the age of 50 (Turton *et al* 1978).

The association of CFA with conditions believed to have an immunological pathogenesis, the development of features of immune dysregulation and the description of family clusters suggests that CFA may have an immunologically mediated pathogenesis following an initial trigger in susceptible individuals. The relative contribution of genetic and external factors is however unclear, particularly as there have been reports of twins developing the disease concurrently despite geographical separation from birth (Hance and Crystal 1983).

It is recognised that infections of the lung by atypical organisms, including viruses, may cause acute episodes of pneumonitis which may result in subsequent chronic scarring (Spencer 1985). This has suggested that CFA may follow an initial, possibly viral, infective trigger. Three possible mechanisms have been postulated whereby a viral infection might result in persistent inflammation and damage.

Firstly, persistence of a virus in the lung may occur with viral antigen expression on host cells resulting in a continuing immunological reaction. Such a process could be analogous to the development of chronic active hepatitis and subsequent cirrhosis of the liver in some patients with persistent Hepatitis B carriage (Millward-Sadler 1987). An association

between CFA and some forms of chronic liver disease has been described (Turner-Warwick 1968) and studies in Japan (Uehda *et al* 1992) and Italy (Meliconi *et al* 1994), but not the UK (Irving *et al* 1993), have suggested an increased incidence of circulating antibodies to Hepatitis C virus in CFA patients. A possible association between CFA and the Epstein-Barr Virus (EBV) has been also suggested following the demonstration of elevated titres ($>1:160$) of antibody to EBV in bronchoalveolar lavage fluid (BAL) obtained from patients with CFA compared to controls (Vergnon *et al* 1984). A recent study has also found evidence of EBV replication in type II alveolar epithelial lining cells in patients with CFA but not controls (Egan *et al* 1995).

A second possible mechanism where a virus is recognised as being able to trigger a persistent inflammatory reaction involves the development of a cross reactive immune response where antibodies or cytotoxic T lymphocytes primed by the virus recognise and interact with endogenous antigens. Such a mechanism has been proposed in viral myocarditis (Rose 1991). The probability of developing cross reacting immune responses is dependent on the epitopes on the virus to which an individual responds and this in turn is dictated by their genetic makeup. Thus such a mechanism would fit with the described family clusters and might explain how even a commonly occurring infective agent could produce such a disease in a few individuals.

Thirdly cellular damage in the lung as the result of a triggering viral infection may result in the exposure of previously sequestered self-antigen and the development of a secondary autoimmune response. Such a mechanism has been postulated to result in the generation of antibodies to thyroglobulin in Hashimoto's autoimmune thyroiditis (Vladutiu 1990). Despite such proposed models serological studies aimed at establishing a viral aetiology have been consistently negative (Herbert *et al* 1962, Stack *et al* 1965, Marks 1967, Stack *et al* 1972).

Epidemiological studies in the UK (Scott *et al* 1990) have also suggested that exposure to industrial dusts and agents might play a role in the development of CFA. Many industrial chemicals and agents are recognised as being capable of inducing an immunological response (Patterson 1982). Such interactions are recognised as being capable of inducing an immunological response to endogenous proteins via a haptencarrier effect (Stein-Streilein *et al* 1987). Previous animal studies have also indicated that even with non-haptogenic agents which result in lung fibrosis,

such as bleomycin, a cellular response to collagen may be generated (Schrier *et al* 1982). Similar features have also been shown in patients with pulmonary fibrosis (Kravis *et al* 1976) although the significance of this to the pathogenesis of the disease is unknown.

Roggli (1991) found in a series of 24 cases of diffuse interstitial fibrosis examined by scanning electron microscopy, 2 cases which were thought to have CFA on histopathological grounds, with lung fibre counts sufficiently elevated to raise the possibility of asbestosis. In addition it is recognised one of the patterns of lung fibrosis that may develop in patients exposed to hard metal dusts such as iron, cadmium, tungsten and aluminium is histologically identical to CFA (Roggli and Schburne 1993). If this were the case, however, the epidemiology of CFA would be predicted to show a male preponderance reflecting workplace exposure with a possible increasing female incidence reflecting changes in working practices since the 1939-45 war. The epidemiological data does not appear to support such a hypothesis.

If exposure to reactive chemical species has any role in the aetiology of CFA then it may be more likely to be an agent present in the general environment to which exposure may be increasing. Cigarette smoke is recognised to contain highly reactive chemicals which may be capable of interacting with tissue proteins and has been implicated in the pathogenesis of another autoimmune inflammatory condition, throboangiitis obliterans (Buerger's Disease) (Pappa *et al* 1992). Smoking was first suggested as a cause of pulmonary fibrosis by Auerbach *et al* (1963, 1974) and its synergistic effect on asbestos related fibrosis is recognised (Weiss 1984). Carrington *et al* (1978) found a high incidence of smoking in patients with CFA . 90% of patients with UIP, but only 71% of patients with DIP had a smoking history of greater than 10 pack years. de Cremoux *et al* (1990) analysed the clinical feature of CFA in smokers and non-smokers and found that smokers tended to have a more aggressive onset and higher cell counts in their bronchoalveolar lavage. No difference in survival was however found between the two groups. Thus while smoking appears to be common in patients with CFA and has a recognised association with the development of a non-specific pattern of pulmonary scarring, a link with CFA is not apparent.

The aetiology of CFA therefore remains unclear. Investigative studies to examine viral infections, workplace exposure and other possible agents are significantly hampered by the gradual onset of symptoms and the

probable long lag time from the initial event until presentation. This is further compounded in that the immunological and pathological features of CFA that can be studied may be the result of a secondary autoimmune process rather than the initial trigger. This also raises the possibility that the clinico-pathological condition recognised as CFA might be triggered by a variety of potential agents which share some common ability to stimulate a secondary autoimmune reaction in susceptible individuals.

1.10. Pathogenesis of Cryptogenic Fibrosing Alveolitis.

Although the aetiology of CFA remains unclear the pathogenesis appears to involve a persistent inflammatory alveolitis (Crystal *et al* 1984 and 1991) which is believed to be the result of an ongoing immunologically mediated reaction (Campbell *et al* 1985), possibly with autoimmune features, associated with the proliferation of pulmonary mesenchymal cells and regeneration of the alveolar epithelium (Hammer 1993).

Histopathologically CFA is characterised by the development of an alveolitis principally composed of macrophages, lymphocytes and plasma cells with variably increased numbers of neutrophils, eosinophils and mast cells (Lamb 1990, Katzenstein and Askin 1990, Hammer 1983). Bronchoalveolar lavage studies have supported the concept of an inflammatory alveolitis in CFA and have shown increased numbers of lymphocytes, macrophages, neutrophils and eosinophils the lung (Reynolds *et al* 1977, Haslam *et al* 1980 and Cherniack *et al* 1990). Immunohistochemical studies have indicated that the majority of the lymphocytes are T cells with both CD4 and CD8 positive subsets being present (Campbell *et al* 1985, Kradin *et al* 1986). Discrete aggregates of B lymphocytes have also been identified in biopsies although germinal centre formation appears rare (Campbell *et al* 1985, Haslam 1990, Wallace *et al* 1994).

The alveolitis is believed to be the primary disease process as it precedes the development of scar tissue rather than arising as a consequence of it (Crystal *et al* 1991). Evidence for this has been obtained by demonstrating the presence of a lymphocytic alveolitis in patients with connective tissue disorders such as rheumatoid arthritis and systemic lupus erythematosus (Wallaert 1990) in the absence of detectable fibrosis. Reports of similar findings in close relatives of patients with CFA have also been

published (Bitterman *et al* 1986) although the significance of this is uncertain given the rarity of family clusters.

Ultrastructural studies (Corrin *et al* 1985) indicate that in CFA development of the alveolitis appears associated with alveolar epithelial injury. This is characterised by type I alveolar epithelial lining cell swelling and necrosis with reduplication of the basement membrane and fraying of underlying collagen. Swelling of endothelial cells has also been observed but appear less marked than that noted in the epithelium. The mechanism of injury is not fully understood but the current belief is that the damage to epithelial cells is principally the result of reactive oxygen species, proteases and other inflammatory mediators liberated as part of an immunological response to unidentified antigen (Crystal *et al* 1991).

Macrophages lavaged from the lungs of patients with CFA release exaggerated amounts of superoxide anion and hydrogen peroxide (Cantin *et al* 1987a, Strausz *et al* 1990) both of which are capable of causing tissue damage. In addition increased amounts of myeloperoxidase can be detected in BAL fluid from patients with CFA (Cantin *et al* 1987a). This enzyme is known to be contained in neutrophils and can convert hydrogen peroxide to a toxic hypohalide radical. *In vitro* experiments with alveolar epithelial cells have shown that co-culture with either recovered inflammatory cells or BAL fluid is cytotoxic and that this effect is synergistic (Cantin *et al* 1987b). *In vitro* this mechanism may be further exacerbated by a reduction in the antioxidant glutathione (GSH) which normally protects the lower respiratory tract from oxidant injury (Cantin *et al* 1987b). For reasons that are not clear there appears to be a four fold decrease in GSH levels detectable in BAL from patients with CFA (Cantin *et al* 1989).

Macrophages and neutrophils also release proteolytic enzymes and the apparent demonstration of collagen fraying in CFA suggests that they may have some role (Corrin *et al* 1985). Increased amounts of collagenase but not elastase have been demonstrated in BAL fluid from patients with CFA (Gadek *et al* 1979). It has been hypothesised that neutrophil elastase may be rapidly inhibited by anti-proteases in the lung (Crystal *et al* 1981) while in contrast the alveolar surface appears to have little protection against collagenolytic enzymes, even though they are usually regarded as less potent in terms of their ability to produce tissue damage (Montano *et al* 1989). An important role for these agents in the disease process is certainly

suggested by the degree of destruction and collapse that occurs within the acinar unit in CFA (Myers and Katzenstein 1988, Burkhardt 1989).

Other inflammatory mediators that may have a role in producing epithelial injury have also been suggested. Mast cell derived histamine (Rankin *et al* 1987) has been detected in increased concentration in BAL from patients with CFA as has eosinophil cationic protein (Hallgren *et al* 1989). The latter in particular is known to be a potent toxin to epithelial cells and may be responsible for much of the epithelial necrosis observed in asthma (Cotram *et al* 1989).

Immunofluorescent techniques have identified the presence of immune complexes in lung biopsies from some patients with CFA (Nagaya *et al* 1969 and 1973, Turner-Warwick *et al* 1971, Hogan *et al* 1978, Schwartz *et al* 1978, Dreisen *et al* 1978, Pratt *et al* 1979). Such findings have been confirmed in studies utilising BAL (Hunninghake *et al* 1981, Dall'Aglio *et al* 1988). The predominant immunoglobulin subclass detected was IgG (Nagaya *et al* 1973, Dreisen *et al* 1978, Dall'Aglio *et al* 1988) although both IgM and IgA have been described (Nagaya *et al* 1969, Pratt *et al* 1979). The complexes were usually within alveolar septa (Nagaya *et al* 1973, Dreisen *et al* 1978, Pratt *et al* 1979) although perivascular deposits have also been noted in some studies (Turner-Warwick *et al* 1971, Dreisen *et al* 1978).

Theoretically these complexes could arise locally in the lung due to the interaction of an antibody with either an endogenous or a persistent exogenous antigen. Alternatively they may be simply deposited in the lung as part of a systemic circulating immune complex disease following their generation elsewhere. Circulating immune complexes have been described in some studies (Haslam *et al* 1979, Crystal *et al* 1984) although the nature of the antigen in the complex is unknown. It is, however, recognised that systemic immune complex diseases are commonly associated with renal injury which is rarely seen in CFA (Turner-Warwick 1974) and this may therefore suggest that the antigen is pulmonary in nature.

The presence of immune complexes in tissue is recognised as being capable of inducing lung injury (Henson *et al* 1979, Daniele 1981) and suggests that this might represent another possible mechanism whereby immunologically mediated damage to the alveolar epithelium could occur. This concept has been supported by observations that complex deposition appears to correlate with areas of cellular active disease and is absent from end stage fibrotic lung (Nagaya *et al* 1969, Dreisen *et al* 1978, Turner-

Warwick *et al* 1971). In one case complexes were detected early in the course of the disease in a biopsy but were not detectable by the time the patient came to autopsy (Pratt *et al* 1979). This relationship of immune complexes to disease activity may explain the variability in the proportions of cases in which complexes have been detected (30-90%).

The epidemiological and aetiological data suggesting a possible autoimmune pattern for the pathogenesis of CFA and the presence of immune complexes in many of these patients has prompted the search for autoantibodies to lung antigens in CFA that might result in complex formation. Evidence for a local humoral immune response in the lungs of patients with CFA has been described (Campbell *et al* 1985, Crystal *et al* 1984) and elevated levels of IgG in BAL demonstrated (Cherniack *et al* 1990) which may be locally produced (Hance *et al* 1988). Studies using indirect immunofluorescent techniques on cryostat section of CFA lung tissue and patient sera have attempted to identify the presence of autoantibodies to lung antigens (Turner-Warwick and Doniach 1965, Ehringer 1991). Such studies have, however, been inconclusive due to high levels of background staining. This has resulted from non-specific interaction between free and cell bound immunoglobulin in the tissue and the fluorescently labelled anti-human immunoglobulin employed in the detection system. A further group claimed to have identified antibodies in CFA patient plasma which recognised isolated rat type II epithelial cells (Baumgartner *et al* 1987) in culture but the frequency of positivity in the patient group (2/10) was not significantly different from the controls (0/11).

The pathogenesis of the immunological injury in CFA is thus unclear and may be multifactorial involving cellular and humoral components of the immune system. The mechanisms of lung injury that have been described, however, cannot explain the unusual and focal nature of the disease process in the lung (see section 1.4). This suggests that there may be local features in these peripheral areas which in some way 'permit' injury to occur within different acini at different times. This pattern of disease also suggests that CFA may be the result of repeated episodes of acute lung injury with subsequent organisation and healing by fibrosis. The regulation of the rate or frequency of injury is unknown but as with other immune mediated diseases such as rheumatoid arthritis it seems possible that disease 'flare ups' may be associated with increased symptoms and a stepwise reduction in lung function rather than a slow gradual decline. This may explain the

clinical observation that the disease course is unpredictable and that patients may show a rapid deterioration after being, apparently, stable for many months or even years (Hunter *et al* 1979).

Recently it has become increasingly apparent that the resident, non-immune, cells of the lung may play important effector roles in the pathogenesis of CFA. These cells do not merely respond in a passive manner to the inflammatory process but interact with the inflammatory cells and may themselves be capable of maintaining the immune process and promoting fibroplasia. The role of these different cells in the inflammatory and fibrogenic processes in CFA and the effects of their interactions with the humoral components remains poorly understood but are reviewed below.

1.10.1 Macrophages.

Histological (Hammer 1993) and BAL (Cherniack *et al* 1990) studies have both indicated an increase in interstitial and alveolar macrophage cell populations which are believed to be brought about by recruitment of bone marrow derived blood monocytes and local proliferation (Hoogsteden *et al* 1989) although the relative contribution of each is unclear. Recruitment of monocytes and neutrophils (see section 1.10.2) to the lung is believed to be mediated by a group of cytokines referred to as the chemokines (Miller and Krankel 1992). This family of cytokines includes interleukin 8 (IL8), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 and 2 (MIP-1 and 2). MCP-1 and MIP-1 and 2 are believed to be the principal chemoattractant factors for monocytes and macrophages and IL8 for neutrophils in a variety of inflammatory lung diseases (Streiter 1994, Driscoll 1994 Personal communications). Studies have indicated that MCP-1, IL8 and MIP-1 and 2 are produced by a range of cells in CFA lung tissue including macrophages, hyperplastic type II epithelial cells, and fibroblasts (Lynch *et al* 1992, Standiford *et al* 1991 and 1993, Rolfe *et al* 1991 and 1992, Iyonaga *et al* 1994). BAL studies have also demonstrated elevated levels of MCP-1 (Streiter 1994 Personal communication).

This suggests that the accumulation of macrophages and neutrophils may be mediated by these agents, initially derived from resident macrophages and structural cells in the lung and subsequently from recruited, activated macrophages. Release of chemokines, *in vitro*, by cultured fibroblasts and type II epithelial cells is recognised to be upregulated by other macrophage derived cytokines including IL1 and TNF α .

(Streiter 1994 Personal communication) suggesting that macrophage activation may further enhance recruitment from the vascular compartment by structural cells.

The mechanism of macrophage activation in the lung is unknown but may be the result of interactions with immune complexes which, as discussed above, have been demonstrated in the alveolar walls of some patients with CFA (see section 1.10). Studies of alveolar macrophages derived by BAL from patients with CFA have indicated a reduced number of IgG Fc receptors on their surface (Hunninghake *et al* 1981) compatible with prior binding and internalisation of immune complexes. *In vitro* studies have indicated that macrophages activated by immune complexes spontaneously release mediators which are chemotactic for neutrophils (Hunninghake *et al* 1980). Lynch *et al* (1992) has demonstrated increased IL8 mRNA in alveolar macrophages derived from CFA patients suggesting that these mediators may be chemokines which as discussed above are thought to be important chemotactic signals for both monocytes and neutrophils. This suggests the presence of a possible positive feedback loop where recruitment and activation of monocytes leads to further cellular recruitment maintaining the inflammatory process.

Macrophages also appear to have a prominent role in the induction of mesenchymal proliferation that characterises the development of fibrosis in CFA. Platelet derived growth factor (PDGF) is the most potent of the macrophage growth factors in its ability to attract mesenchymal cells and stimulate them to enter the growth cycle (Crystal *et al* 1991). In CFA alveolar macrophages release up to fourfold more PDGF than controls and immune complexes are among the most potent stimulators of PDGF release *in vitro* (Martinet *et al* 1987).

Alveolar macrophages also produce fibronectin (Crystal *et al* 1991), an extra-cellular matrix protein which has been demonstrated to stimulate mesenchymal cell proliferation (Bitterman *et al* 1983). In patients with CFA alveolar macrophages express increased amounts of fibronectin mRNA transcripts (Rennard *et al* 1981) and increased levels of the protein can be detected in lavage fluid (Rennard and Crystal 1982). The effects of PDGF and fibronectin on mesenchymal cell proliferation at least *in vitro* appears to be enhanced by the peptide growth factor IGF-1 (insulin like growth factor-1 or somatomedin C) (Crystal *et al* 1991). This peptide appears to be spontaneously released by alveolar macrophages from patients with CFA

(Nagaoki *et al* 1990) although its *in vitro* contribution to the mesenchymal response is not understood.

Transforming growth factor- β (TGF β) is also produced by alveolar macrophages (Yamauchi *et al* 1987, Khalil *et al* 1989) and increased amounts have been detected in BAL fluid from patients with CFA (Yamauchi *et al* 1988). The biological role of TGF β in the production of the mesenchymal response is unclear. Originally it was thought that *in-vitro* it may decrease mesenchymal cell proliferation (Roberts *et al* 1985), however, recent studies have indicated that in association with other factors such as PDGF it may stimulate division and possibly differentiation to a more "aggressive" phenotype (Gauldie 1993). What is certain is that TGF β up regulates the genes for collagen (Fine and Goldstein 1987) and fibronectin (Roberts *et al* 1988) thus stimulating the production of the extracellular matrix and it has been identified at sites of scarring in the human lung (Broekelmann *et al* 1991, Khalil *et al* 1991). Recent data has indicated that administration of monoclonal antibodies to TGF β blocks the development of fibrosis following the administration of bleomycin (Giri *et al* 1993) and this has focused attention on TGF β as one of the principal mediators of scarring in many tissues and therefore a possible target for therapeutic intervention (Laurent *et al* 1993).

The data reviewed above suggests that the macrophage plays a potentially critical role in CFA and they are considered by many to be the central effector cells in maintaining the inflammatory process and driving the mesenchymal cell proliferation that is evident in CFA (Crystal *et al* 1991).

1.10.2 Neutrophils.

The release of chemotactic factors by macrophages (Lynch *et al* 1992) and other structural cells within the lung (Rolfe *et al* 1991) are thought to be the major factors responsible for recruiting neutrophils to the lung. While neutrophils are often not prominent histologically in CFA tissue, studies of inflammatory cells extracted from biopsy tissue and BAL have both shown significant numbers to be present (Reynolds *et al* 1977, Haslam *et al* 1980, Hunninghake *et al* 1981). Their distribution also appears to be rather focal (Hammer 1993) within the tissues and the reasons for this, given the apparently rather ubiquitous presence of activated macrophages, is unclear. Pathologically these cells are most often evident in areas of active disease

with associated alveolar leak (Hammer 1993) suggesting that they are found at sites of epithelial injury.

It is recognised that immune complexes are more often identified at such sites in CFA (Nagaya *et al* 1969, Dreisen *et al* 1978, Turner-Warwick *et al* 1971). This raises the possibility that these may influence the distribution of neutrophils in the lung through their known chemotactic function (Daniele 1981). The interaction of neutrophils with immune complexes is recognised to promote cellular activation with release of lytic enzymes and reactive oxygen species which are thought to be important in producing the epithelial injury observed in CFA (see section 1.10). In many respects the neutrophil is a more potent source of these factors than the macrophage (Hance and Crystal 1983) and in other pulmonary conditions such as the adult respiratory distress syndrome (ARDS) is recognised as being capable of inducing widespread acute lung injury (Bartels and Reale 1991).

Recruitment of macrophages and neutrophils into the lung in CFA is believed to be mediated by interaction of adhesion molecules on the cell surface with ligands on endothelial cells (Shijubo *et al* 1992). $\text{TNF}\alpha$ has the effect of increasing endothelial and epithelial expression of intracellular adhesion molecule-1 (ICAM-1) (Mulligan *et al* 1993) which is the ligand for the CD11/CD18 β 2-integrin expressed on neutrophils and macrophages (Shijubo *et al* 1992). Lavaged alveolar macrophages from patients with CFA are recognised to have increased expression of β 2-integrin (Schaberg *et al* 1993). The importance of this process in the accumulation of these cells and the development of subsequent fibrosis has been illustrated by Piquet *et al* (1990 and 1993) who showed that treatment of mice with monoclonal antibodies to CD11a or $\text{TNF}\alpha$ completely blocked the fibrotic response to intratracheal bleomycin or silica. This indicates that even in toxic models of lung injury recruitment of macrophages and neutrophils appears to be an essential step in the development of lung injury (see sections 1.10) although the relative contribution of each is unclear.

If the neutrophil is the principal effector cell in producing lung damage then elevated number in BAL might be expected to imply the presence of an active alveolitis and therefore a better response to therapy. Several groups have shown using bronchoalveolar lavage that a high neutrophil count is in fact associated with a poor response to immunosuppressive therapy (see section 1.7). The reasons for this are unclear but may result from sampling problems. In active disease the neutrophils producing the damage may be

within the tissue and therefore not sampled by BAL. In established fibrotic disease large honeycomb spaces are characteristic (see section 1.4) and these often contain pus. Thus in fibrotic disease large numbers of neutrophils may be sampled by lavage which are present in the airspace but not participating in the actual disease process.

1.10.3 Eosinophils.

The mechanisms resulting in the recruitment of eosinophils to the alveolitis in CFA are not understood but may relate to factors released from mast cells (section 1.10.4) or a low level response to the potent neutrophil chemotactic agents which are present in the lung. The presence of eosinophils in BAL is, however, characteristic of CFA and may carry similar implications for response to therapy as neutrophils (Haslam *et al* 1980, Turner-Warwick 1987). Patients with CFA are not recognised to show elevated IgE levels or a peripheral blood eosinophilia (Reynolds *et al* 1977) although a recent small study has suggested that atopy may be more common in these patients than in controls (Marsh *et al* 1994). The exact role of eosinophils in the disease process is unclear but they are recognised to have a potent oxidative function (Spry 1981) and increased levels of eosinophil cationic protein have been detected in BAL fluid from patients with CFA (Hällgren *et al* 1989) suggesting that they may have some role in epithelial injury.

1.10.4 Mast Cells.

Increased numbers of mast cells have been demonstrated in CFA lung tissue (Haslam *et al* 1981, Hunt *et al* 1992) and in the lungs of patients with scleroderma (Chanez *et al* 1993). In the latter group the numbers were higher in patients with chest x-ray abnormalities suggesting onset of fibrosis compared with those with normal chest x-rays. Such studies have also indicated that the mast cell numbers are preferentially increased in diseased areas of the lung (Haslam *et al* 1981). In addition the morphological appearances of the mast cells in CFA (Hunt *et al* 1992), coupled with evidence of increased concentrations of histamine and tryptase in BAL fluid (Haslam *et al* 1981) has suggested that the cells are activated and degranulating.

The role of mast cells in the pathogenesis is unclear but in experimental animal models where degranulation has been induced a fibrotic

response can be demonstrated (Norby 1981). Inhibition of mast cell degranulation may inhibit experimentally induced fibrosis (Walker *et al* 1987) suggesting a critical role for mast cell derived factors. Murine mast cells are recognised to produce a range of cytokines including IL1, IL3, IL4, GM-CSF and TNF α although less is known about the spectrum produced by human mast cells (Galli 1991). This suggests that the mast cell may potentially function as a source of cytokines which promote both the inflammatory and fibrogenic response in the lung. *In vitro* studies have indicated an almost symbiotic relationship between mast cells and fibroblasts each promoting the others growth (Druvefors *et al* 1988). This may involve the endocytosis of mast cell granules by adjacent fibroblasts in a process called 'transgranulation' (Greenberg and Burnstock 1983, Subba Rao *et al* 1983). The significance of this process is uncertain but theoretically provides for a very efficient and potent method of fibroblast stimulation by mast cell derived growth factors.

1.10.5 Fibroblasts and the Extracellular Matrix.

The regulation of the fibroblast function in CFA remains poorly understood but is important as the development of scar tissue is responsible for the ultimate morbidity and mortality. It is likely to involve many factors with positive and negative effects which themselves may be enhanced or diminished by other agents thus creating potential autocrine feedback loops to potentiate the process. Much of the data on such effects has been derived from *in vitro* experiments in which purified or partially purified reagents were applied to fibroblasts in isolation. The relevance to the pathogenesis of CFA of some of these findings must therefore be in doubt as they represent simplification of the *in vivo* disease process.

In CFA the development of scar tissue appears to result from an increase in the number of mesenchymal cells and deposition of increased amounts of extracellular matrix proteins, especially collagen. This appears secondary to an increase in mesenchymal cell proliferation in response to the production of a variety of growth factors derived from inflammatory and resident lung cells (Gauldie 1993). Regulation of mesenchymal cell proliferation and deposition of extracellular matrix proteins is likely to be the result of interactions of agents some of which may be synergistic (as discussed in section 1.10.1) and others antagonistic.

Prostaglandin E (PGE) has been shown to inhibit mesenchymal cell proliferation and synthesis of collagen (Ozaki *et al* 1987) *in vitro* and BAL fluid from patients with CFA show reduced amounts of PGE (Crystal *et al* 1991) suggesting that loss of inhibitory factors as well as the presence of growth factors may be important. This suggests the possibility that the pattern of immune activation in CFA may favour a fibroblastic response. $\text{INF}\gamma$ is recognised to inhibit fibroblast proliferation and collagen production *in vitro* (Pfeiffer *et al* 1979, Duncan *et al* 1985) and Prior and Haslam (1992) has shown that serum levels of this cytokine are normal in most patients with CFA unlike those with sarcoidosis. Further they observed that in those who did have elevated levels disease progression appeared slower, suggesting a possible *in vivo* inhibitory effect. Recent personal studies using immunohistochemistry have indicated that the majority (around 60%) of infiltrating inflammatory cells in CFA lung biopsies are positive for IL4 and IL5 while less than 5% are positive for $\text{INF}\gamma$ (Wallace *et al* 1995a). This suggests the presence of a predominantly type II (Th 2 like) pattern of immune reaction (Romagnani 1991, Clerici and Shearer 1994) in CFA and explains the previous paucity of $\text{INF}\gamma$ in the serum of these patients.

The possibility that fibroblasts do not represent a uniform cell population which respond identically to stimulatory and inhibitory signals has also been suggested. Sempowski *et al* (1994) has identified two populations of murine pulmonary fibroblasts which differ in their responses to cytokines *in vitro*. One subset responded to exogenous IL4 by increased proliferation and synthesis of collagen while the other did not. Interestingly the IL4 sensitive population also appeared to be inhibited by $\text{INF}\gamma$ (Sempowski, personal communication). This suggests that the particular subtypes of fibroblasts present in an individuals lung might determine susceptibility to CFA and other fibrotic lung conditions.

The fibroblast in CFA does not, however, appear to be simply an end point effector cell driven by exogenous factors. Recent evidence suggests that they may have a role in promoting both the inflammatory and fibroblastic processes occurring in CFA. Chemokines such as IL8 and MCP-1 (Rolfe *et al* 1991 and 1992) have been shown to be produced by these cells and production, at least *in vitro*, to be enhanced by other macrophage derived pro-inflammatory cytokines such as IL1 and $\text{TNF}\alpha$ (Streiter 1994 Personal communication). Fibroblasts also appear capable of producing their own growth factors such as fibronectin and PDGF (Gauldie 1992) which may act

in an autocrine fashion promoting further proliferation. As will be described below (section 1.9.6) they may also be involved in the regulation of type II epithelial cell proliferation which may also have an important role in the regulation of scar formation.

The exact mechanism of fibrosis in CFA is unclear. Ultrastructural studies have suggested that following loss of epithelial integrity at sites of injury migration of fibroblasts into the lumen occurs, leading to the development of 'intraluminal buds' (Basset *et al* 1986). These buds are composed of parallel-arranged, fibroblast like cells and may progress to fill the alveolus and fuse with adjacent structures. The fibroblastic process may therefore in part result from coalescence of collapsed alveolar septa with the subsequent incorporation of the organising intra-alveolar exudate into the interstitium and re-epithelialisation (Hammer 1993). The fate of the 'bud' after incorporation is unclear but the fibroblasts may continue to proliferate producing a true interstitial fibroblastic process.

This pattern of organising intra-alveolar exudates appears similar to that observed in ARDS (Haselton 1983, Kuhn *et al* 1989) and bronchiolitis obliterans organising pneumonia (BOOP; Epler *et al* 1985). Intriguingly it is recognised that in these conditions intraluminal fibrosis may be reversible with little or no disturbance of the lung architecture. Polunovsky *et al* (1993) has shown that during the resolution phase of patients with ARDS a factor inducing fibroblast apoptosis is detectable in BAL and in patients with BOOP complete resolution with steroid therapy is observed (Epler *et al* 1985). This suggests that while the initial phases of the fibroblastic process in CFA may resemble those observed in other types of lung injury the regulation of subsequent events is very different.

While mesenchymal cell proliferation is an important feature of scarring a marked alteration in the extracellular matrix composition is also characteristic. In the normal lung the small amounts of collagen in the acinar unit are predominantly types I and III in a ratio of approximately 2:1. (Hammer 1993). Raghu *et al* (1985) and Bateman *et al* (1981) found that in early disease type III collagen was dominant and that this was replaced by type I collagen as the disease progressed.

Despite this there is still argument and contradictory data about whether the total collagen content of the lung is increased in CFA. The total collagen content of the normal lung as assessed by dry weight is around 15% (Hammer 1993). Most of this collagen is, however, associated with the

broncho-vascular bundles rather than the alveolated lung. Studies showing no change in total collagen content (Fullmer *et al* 1980) and others showing an increase to around 25% of dry weight have been published (Madri and Furthmayr 1980). Part of this apparent discrepancy may relate to the amount of tissue utilised in the studies and the stage of the disease. In relatively early disease most of the collagen will still be associated with the bronchovascular bundles and although there may be an increase in septal collagen this may not result in an appreciable increase in the total collagen. In later stages of the disease the entire specimen may be scarred and hence the total collagen content will be elevated.

Even less is known about the production and distribution of other, non-collagenous, extracellular matrix proteins which potentially may have roles in the regulation of fibroblast and inflammatory cell migration as well as the development of scar tissue. As discussed above (section 1.9.1), increased levels of fibronectin have been detected in the lungs of patients with CFA and this has been suggested to have a regulatory function on fibroblast proliferation and migration (Bitterman *et al* 1983). Increased levels of vitronectin, a glycoprotein which promotes cell spreading and adhesion, has also been detected in BAL samples from patients with CFA (Pohl *et al* 1991) and may have similar effects to fibronectin in promoting cell migration and proliferation.

Recent personal studies have focused on another extracellular protein, tenascin. Tenascin is a large, 190-240 kDa, extracellular matrix glycoprotein with a hexameric, multidomain structure composed of disulphide-linked subunits (Erickson *et al* 1989). In the embryo it appears to show temporal expression in many developing tissues including breast, lung, tooth and kidney but appears to be absent from most normal adult tissues (Chiquet-Ehrismann *et al* 1986). Production has however been shown in the stroma of some neoplasms (Mackie *et al* 1981, Stamp 1989) and is locally increased during tissue repair (Mackie *et al* 1988). In experimental skin wounds tenascin can be detected in the basement membrane region of the regenerating epithelium and in the subjacent granulation tissue. Following completion of the scarring process tenascin expression ceases in the tissue (Mackie *et al* 1988).

Personal studies (Wallace *et al* 1995b) have indicated that immunoreactive tenascin is not identified in histologically normal lung parenchyma. However in CFA tenascin production can be demonstrated in

minimally damaged alveolar walls and fibroblastic foci associated with hyperplastic type II cells but not in end stage scarred lung. The presence of temporally expressed extracellular matrix proteins in the disease process which may be under local cytokine control (Pearson *et al* 1988, McCachren *et al* 1992, Meiners *et al* 1993) suggests that they may have important, although as yet, poorly understood regulatory functions.

The possibility that inflammatory cells and resident epithelial and mesenchymal cells may be significantly influenced in their behaviour by interaction with the extracellular environment is an area that remains poorly understood. What is increasingly clear, however, is that fibroblasts and extracellular matrix proteins are not merely end effector cells and proteins but probably have important functional and regulatory capacities in the tissue remodeling that occurs in CFA.

1.10.6 Alveolar Epithelial Cells.

The alveolar epithelium, as discussed above, is believed to be the principal site of immunological injury in CFA (see section 1.10). Injury to type I cells has been recognised to result in proliferation of the type II cells which are believed to contain the relevant stem cell compartment (Mason and Williams 1991). Our knowledge of the function and biology of type I alveolar cells (Schneeberger 1991) is limited but more is known about type II cells (Mason and Williams 1991).

Type II cells (see review by Mason and Williams 1991) normally cover around 15% of the alveolar surface and ultrastructurally appear polarised. It is thought that their principal function is the secretion of surface active molecules of which surfactant is the most important. It is, however, recognised that these cells synthesise and secrete other molecules which may be important in lung defence including lysosomal enzymes and various complement factors. In CFA the proliferating hyperplastic type II cells are recognised to show a degree of cytological atypia and on occasion multinucleate forms may be observed (Katzenstein and Askin 1990). The reason for the atypia is unclear. It may represent cellular activation or 'regenerative atypia' as may be observed in other mucosal surfaces which are ulcerated (Yogeshwar and DeLellis 1989). Similar appearances, however, have also been described in virally infected cells (von Lichtenstein 1989) which is of particular interest given the recent evidence for replicating Epstein-Barr virus in such cells (Egan *et al* 1995).



Epithelial cells are capable of producing inflammatory cytokines in response to direct stimulation in a variety of systems. Investigation of the mechanisms responsible for inflammation in the skin in contact dermatitis has indicated that keratinocytes are capable of producing a wide range of cytokines, including IL8 (Howie *et al* 1992), as a primary effect. Experiments involving the application of silica powder, which is known to be intensely fibrogenic *in vivo*, onto the surface of the type II epithelial cell line, A549, *in vitro* results in the production of MIP-2 in the absence of other stimulatory factors (Driscoll 1994 Personal communication). Interestingly the application of titanium oxide, which appears *in vivo* to be inert, did not result in the production of MIP-2. These results suggest that epithelial cells respond selectively to 'irritants' and that production of cytokines may be induced directly by some form of interaction between the agent and the cell.

Immunohistochemical studies have confirmed that hyperplastic type II epithelial cells in CFA are also positive for cytokines such as MCP-1 (Streiter 1994 Personal communication, Lyonaga *et al* 1994), MIP-2 (Driscoll 1994 Personal communication) and IL8 (Streiter 1994 Personal communication) which, as discussed above, appear to be important in the development and maintenance of the alveolitis. Comparison of hyperplastic and metaplastic epithelial cell staining for MCP-1 in CFA with other cases of non-specific fibrosis or bronchiolitis obliterans organising pneumonia (BOOP) revealed much more prominent staining in CFA compared to non-CFA cases (Lyonaga *et al* 1994). The authors suggested that activation of epithelial cells and production of these and other cytokines might be responsible for the apparent relentless progress of CFA compared with other conditions. Recent studies have also indicated that these cells are positive for $\text{TNF}\alpha$ (Piguet *et al* 1993) raising the possibility that an autocrine feedback mechanism may be operating as, at least *in vivo*, $\text{TNF}\alpha$ enhances chemokine production by both macrophages and epithelial cells (Streiter 1994, Driscoll 1994 Personal communications).

These cells also produce factors which, as discussed above, are recognised to be potent agents in the proliferation of mesenchymal cells and production of extracellular matrix proteins by fibroblasts. $\text{TGF}\beta$ (Corrin *et al* 1994) and PDGF (Antoniades *et al* 1990) have been demonstrated associated with hyperplastic type II cells *in vivo* using immunohistochemical techniques. Recently transgenic animals which over express the gene for $\text{TGF}\alpha$ (or epidermal growth factor) in pulmonary epithelial cells have been

shown to develop severe pulmonary fibrosis (Korfhagen *et al* 1994) further supporting the concept that epithelial derived cytokines may have potent effects of fibroblast function.

Type II alveolar cell hyperplasia appears to have a more complex role than simply repair of the epithelial integrity following lung injury. These cells have been demonstrated to have direct intercellular contacts with mesenchymal cells and extracellular matrix proteins (Adamson *et al* 1990). Adamson *et al* (1988 and 1990), in an animal model, has shown that low grade injury with type I cell necrosis is associated with a short lived burst of type II proliferation but fibrosis does not develop. Following more prolonged injury the type II cell proliferative phase was extended and fibrosis ensued. During the periods of maximal cell proliferation the number of epithelial/fibroblast contacts was decreased but later increased as fibrosis developed. This suggests that epithelial injury with delayed repair promotes the development of fibrosis and that contact between epithelial cells and fibroblasts or some extracellular matrix component may regulate type II cell function.

This concept has been supported by studies with keratinocyte growth factor (KGF), a member of the fibroblast growth factor family (Ulich *et al* 1994), which appears to be specific for epithelial cells. KGF is produced by mesenchymal cells (Ulich *et al* 1994) and is upregulated in experimental wound healing (Stasio-Coico *et al* 1993) suggesting that it plays a role in mesenchymal/epithelial cell regulation. KGF has been shown in the lung to be a potent mitogen for type II epithelial cells *in vivo* (Ulich *et al* 1994) and type II derived cell lines *in vitro* (Panos 1994 Personal communication). Studies have shown that instillation of KGF into the lung prior to the administration of hyperoxia or other injurious agents may have a protective effect (Panos 1994 Personal communication). This may be the result of promoting rapid re-epithelialisation of the basement membrane thus inhibiting the fibroblastic response that would otherwise occur as suggested by Adamson *et al* (1988 and 1990).

At sites of scarring most newly synthesised matrix proteins are assumed to be produced by mesenchymal cells but it is recognised that type II epithelial cells may produce fibronectin and a heterogeneous pattern of collagens at least *in vitro* (Sage *et al* 1983, Crouch *et al* 1987, Leheup *et al* 1989). The significance and regulation of extracellular matrix deposition by these cells is unknown but interactions between type II cells receptors for

fibronectin and fibronectin have been demonstrated (Rannels *et al* 1987). Extracellular matrix proteins acting via cell surface integrins are recognised in a number of systems to be capable of modulating epithelial cell division, motility and differentiation by actions on the cytoskeleton (Rahilly *et al* 1991, Salter 1992) and suggests that the extracellular matrix may provide a possible signalling mechanism between cells acting via specific receptors.

These data raise the possibility that injury to alveolar epithelial cells may, under some conditions, set in train a pattern of cytokine production and cellular activation with the potential for autocrine enhancement of both the inflammatory and fibrogenic processes. If the inflammatory process, once initiated, were to result in further epithelial damage or the insult was continuous then extensive lung injury and fibrosis might be predicted with the epithelial cell being a key regulator in the inflammatory and fibrogenic processes.

1.10.7 Lymphocytes and Plasma Cells.

As discussed above CFA has many features which suggest an autoimmune pathogenesis and this implies a lymphocyte mediated response to antigen (see section 1.10). The alveolitis in CFA contains a significant number of lymphocytes and some authors have described it as a T cell alveolitis with apparently equal numbers of CD4 and CD8 cells being detectable in the tissue (Kradin *et al* 1986). B lymphocytes are, however, also frequently observed and may constitute around 10% of the total cell infiltrate (Hunninghake *et al* 1981). They are usually observed forming loose aggregates associated with plasma cells but rarely show germinal centre formation (Campbell *et al* 1985, Kradin *et al* 1986, Haslam 1990, Wallace *et al* 1994).

The nature of the antigen to which the response may be occurring is as yet unknown although the possibility of a role for T lymphocytes sensitised to collagen has been suggested in scleroderma and bleomycin-induced pulmonary fibrosis (Schrier *et al* 1982, Kravis *et al* 1976). However, as discussed previously the majority of patients with CFA and mice treated with bleomycin do not develop multi-system disease. The significance of such auto-reactive T cells is therefore difficult to interpret especially given the realisation that all individuals have a wide range of T cells and antibodies directed against self antigens including collagen (Avrameas 1991).

Systemic evidence indicating T cell activation has been demonstrated in CFA (Meliconi *et al* 1990) as indicated by elevated levels of soluble interleukin-2 receptor (IL2r) and soluble CD8 in serum and the induction of pulmonary fibrosis, at least in experimental models, appears to require the participation of lymphocytes. Experiments conducted with nude mice have indicated that the development of fibrosis requires an immune system even when the injury is toxic, as with bleomycin, and not primarily immunological in nature (Schrier *et al* 1983). This suggests that factors released from lymphocytes may be important in the activation of macrophages or the proliferation of mesenchymal cells and the production of scarring (Li *et al* 1992).

Lymphocytes may play a role in the pathogenesis of the mesenchymal proliferation in CFA. Selman *et al* (1990) showed that media conditioned by T lymphocytes recovered from CFA lung biopsies did not cause significant fibroblast proliferation but induced collagen synthesis. This is a recognised pattern of response to TGF β in the absence of other factors (see section 1.10.1) and suggests that lymphocytes as well as macrophages may be a source of this cytokine in CFA. As discussed above other lymphocyte cytokines such as IL4 and INF γ have also been suggested as possible regulatory factors for the fibroblast proliferation.

In addition to T cells having a function in cell mediated responses the possibility that T cell activation in CFA reflects a function of a local humoral response has also been considered. As discussed previously studies have suggested the presence of a local humoral immune response in the lungs of patients with CFA. Personal studies have suggested that the B lymphocyte aggregates in CFA may represent a MALT pattern of reaction in the lung (Wallace *et al* 1994) and others have demonstrated an increase in B lymphocyte growth factors in BAL (Emura *et al* 1990) suggesting that the aggregates of B lymphocytes, despite their lack of germinal centre formation, may be functioning to process antigen and promote plasma cell differentiation. The predominance of a type 2 (Th2 like) cytokine environment would also support this hypothesis of an antibody mediated process in CFA (Wallace *et al* 1995).

Haslam (1990) suggested that increased numbers of B lymphocyte aggregates in biopsy specimens was associated with a poor prognosis. This was further addressed by Cathcart *et al* (1987) who studied the helper function of peripheral T lymphocytes in patients with CFA using an *in vitro*

antibody synthesis assay. Eight out of fourteen patients studied had higher levels of helper function than controls and these cases were associated with the presence of neutrophilia in BAL. Those with low neutrophil BAL counts had normal or low helper function. As has already been discussed above there is evidence of humoral immune activity and dysregulation with an apparent increase in the local production of immunoglobulin in the lungs of patients with CFA (Hance *et al* 1988) which may be a reflection of the above observation.

While information on the role of lymphocytes in the pathogenesis is thus limited the data that exists suggests a local reaction to as yet undefined antigen(s) and a subsequent regulatory role in macrophage and fibroblast function via cytokine production. In addition there is evidence to suggest that CFA may be associated with a humoral immune reaction in the lung supporting the concept that autoantibodies may have a role in the pathogenesis.

1.11. Cryptogenic Fibrosing Alveolitis: Disease or Syndrome?

The idea that CFA is one condition has been supported by Crystal *et al* (1991) on the basis that the clinical, pathological and immunological features show reasonable homogeneity. It is, however, possible to hypothesise that rather than being one condition, CFA is more of a single syndrome characterised by a particular pattern of response to injury in the lung. This model would suggest that CFA may have a number of aetiologies which result in a common mode or site of injury to the lung and a stereotyped response which is clinically recognised as CFA. What is certainly apparent to pathologists is that the lung has a very limited pattern of response to injury and it would therefore not be surprising that diverse stimuli could result in an apparently identical disease process. In this respect it is interesting to note that in Corrin *et al* (1985) ultrastructural studies which identified the alveolar epithelium as the site of immunological injury in CFA identical features were observed in patients with known asbestosis.

With the exception of patients with connective tissue disorders the early phases of CFA are not available for study. By the time investigations are performed, features that might point to diverse aetiologies have been obliterated by the development of a common disease process which proceeds inexorably to the death of the patient. The features and properties

that such aetiological agents might share which result in the development of such a pattern of progressive pulmonary disease are, however, unknown.

1.12. Autoantibodies To Lung Antigen(s) in Cryptogenic Fibrosing Alveolitis: a Unifying Hypothesis?

Much of the data gathered on the pathogenesis of CFA strongly suggests that it has autoimmune features and although no auto-antigen has been detected the presence of immune complexes is well established. From the review of the literature on CFA it is apparent that the presence of immune complexes in the lung could account for most of the patterns of epithelial injury and immune cell activation that is known to occur. As CFA is not associated with renal disease the possibility that the antigen involved is pulmonary seems more likely than the alternative hypothesis that the immune complexes are deposited as part of a systemic immune complex disease. This suggests that CFA must be associated with a humoral immune response and the development of autoantibodies to antigen(s) in the lung.

If CFA is regarded as a syndrome rather than a single disease entity then it is possible to hypothesise that the development of antibodies to lung antigen following an initial trigger with subsequent persistent epithelial injury may be the common feature. Injurious agents which did not result in the development of autoantibodies would be predicted to result in different patterns of disease which, furthermore, might be relatively self limiting as no autoimmune sequelae would be invoked. Such a mechanism might also predict that exposure of some individuals to the agents would result in the development of CFA whereas others would not due to the diversity of the genetic regulation of the immune response.

The key to this hypothesis is that patients who develop CFA do so because they develop autoantibodies to lung antigens as a consequence of an injurious environmental trigger. This hypothesis has been proposed for over 30 years (Burrell 1963, Turner-Warwick and Doniach 1965) but has failed to be developed due to the inability to demonstrate the presence of lung specific autoantibodies in patients.

In this thesis I have re-addressed the question of whether patients with CFA have circulating autoantibodies to lung antigens. These studies have been conducted on a pathologically well defined group of patients with CFA whose diagnoses have been confirmed by open lung biopsy. Modern cell biology and immunological techniques were then applied using the

clinical samples available to address this question. I will present the results of experiments designed to demonstrate the presence of such autoantibodies and to elucidate the distribution and nature of the autoantigen. In addition the possible pathological significance that these autoantibodies may have in CFA will be examined.

In the period January 1983 to December 1983 121 patients underwent thoracic biopsy for the investigation of diffuse pulmonary disease. These patients were referred to the Regional Thoracic Unit at the Royal Infirmary, Edinburgh from Chest Physicians in the South East of Scotland serving a population of around 1 million. The frequency with which this biopsy was performed on patients presenting with diffuse lung disease was steadily over the period studied (Table 2.1). The reasons for this rise in the biopsy rate included the introduction of bronchoalveolar lavage (Carrington *et al* 1984) and a change of attitude to the investigation of this group of patients by chest physicians.

2.2.1. Summary of Objectives

All the biopsies obtained in this period were either reported or re-examined by Dr. David Lamb, Reader in Pathology, in the Department of Pathology, Edinburgh University. In many instances the results of the histology with the referring physician and presentation at subsequent clinical meetings. A breakdown of the diagnoses obtained was made and is indicated in Table 2.2. CFA represented by far the most frequent diagnosis obtained at biopsy, although the number of biopsies interpreted as normal was also high. The proportion of cases that were histologically diagnosed as CFA was noted (Table 2.1).

CHAPTER 2.

MATERIALS AND METHODS

2.1 Patients Undergoing Open or Thoracoscopic Lung Biopsy (1990-94)

2.1.1 Biopsies Performed.

In the period January 1990 to December 1993 121 patients underwent open or thoracoscopic lung biopsy for the investigation of diffuse pulmonary disease. These patients were referred to the Regional Thoracic Unit at the City Hospital, Edinburgh from Chest Physicians in the South East of Scotland serving a population of around 1 million. The frequency with which lung biopsy was performed on patients presenting with diffuse lung disease rose sharply over the period studied (Table 2.1). The reasons for this relate to the local introduction of thoracoscopic techniques (Carnochan *et al* 1994) and a change of attitude to the investigation of this group of patients by Chest Physicians.

2.1.2 Diagnoses Obtained.

All the biopsies performed in this period were either reported or subsequently reviewed by Dr. David Lamb, Reader in Pathology in the Department of Pathology, Edinburgh University. In many instances there was discussion of the histology with the referring physician and presentation at clinico-pathological meetings. A breakdown of the diagnoses obtained over this period is indicated in Table 2.2. CFA represents by far the most frequent diagnosis obtained at biopsy, although as the number of biopsies increased a parallel decrease in the proportion of cases that were histologically diagnosed as CFA was noted (Table 2.1).

Table 2.1

Number of open or thoracoscopic lung biopsies performed in South East Scotland in the period 1990-93 and the proportion of the total that were reported as showing the histological features of CFA.

Year	Total No. of Biopsies Performed	Total No. of Biopsies Reported as CFA (%)
1990	9	8 (89)
1991	16	9 (56)
1992	46	16 (35)
1993	50	20 (40)

Table 2.2

Diagnoses obtained on 121 open or thoracoscopic lung biopsies performed between 1990 and 1993.

Histological Diagnosis	Number of Cases (%).
CFA	54 (44.6)
Sarcoid	9 (7.4)
Extrinsic Allergic Alveolitis	11 (9.1)
Bronchiolitis Obliterans, Organising Pneumonia	8 (6.6)
Malignancy	7 (5.8)
Diffuse Alveolar Damage Syndrome/ Organising Pneumonias	9 (7.4)
Pneumoconioses	3 (2.5)
No Histological Abnormality	1 (0.8)
Non-specific Features	9 (7.4)
Miscellaneous*	10 (8.3)

* includes amyloidosis, lymphangioleiomyomatosis, pulmonary hypertension, lymphocytic interstitial pneumonitis and pulmonary infarction.

2.1.3 Histological Criteria for the Diagnosis of CFA.

CFA was diagnosed according to the recognised histological criteria which have been previously described (section 1.4) and recently reviewed in detail (Hammer 1994; Katzenstein and Askin 1990).

The following histological features were considered to be characteristic of CFA and were required to be present in order to make the diagnosis.

1. A disease process showing sub-pleural accentuation and relative sparing of centri-acinar structures and bronchioles.
2. A highly variegated lung architecture often including the entire spectrum from normal alveolar walls to end stage fibrotic lesions in the same tissue section.
3. Variable pleomorphic interstitial cellular infiltrate composed principally of monocytes and lymphocytes with relatively few granulocytes.
4. Variegated epithelial lining cells ranging from normal alveolar epithelial lining cells in undamaged areas of lung to large rounded, hyperplastic type II cells in diseased areas. Focal ciliated columnar, goblet and squamous cell metaplasia may be present particularly in severely damaged areas.
5. Variable numbers of cells in the airspaces which are predominantly macrophages although focally a few lymphocytes and granulocytes may be evident.
6. Occasional foci of proteinaceous exudates or organising intraluminal exudates often associated with areas of active alveolitis.

The presence and degree of interstitial fibrosis and honeycomb structures were noted but were not in themselves regarded as diagnostic in the absence of the above features. In some cases evidence of a more acute, possibly infective process, was superimposed on the above features. This usually was identified by the presence of a bronchiolitis with increased amounts of organising intraluminal exudates which tended to have a more proximal position in the acinus. Such cases were still regarded as being CFA although the possibility of recent infection was commented on in the report.

2.2 Patient Samples

2.2.1 Processing of Routine Biopsy Material.

All the biopsies were of a good size, measuring at least 3 cm in maximum dimension. They were inflated in theatre as soon as practical after removal from the patient by transpleural injection of 10% formalin to improve the appreciation of lung architecture and give rapid fixation of the tissue. The biopsy was then sliced and processed through paraffin wax for routine histology. The tissue blocks were stored in the archives of the Department of Pathology.

2.2.2 Collection and Processing of Material for Cryostat Sections.

Tissue was removed from biopsy specimens prior to fixation and placed in OCT (Miles Inc., USA) freezing compound. The tissue was then snap frozen in liquid nitrogen and stored at -70°C . $6\mu\text{m}$ sections were cut, acetone fixed and stored at -20°C prior to use.

2.2.3 Collection and Processing of Material for Protein Extraction.

Tissue was removed from the specimen prior to fixation and placed in a non-ionic NP40 lysis buffer [5mM tris/HCl pH 8.0 containing 1% Nonidet P-40 NP40 (BDH Ltd., UK)]. The material was then snap frozen in liquid nitrogen and stored at -70°C .

2.2.3.1 Preparation of Protein Extracts from Biopsy Tissue.

Protein extracts from tissue were produced using a variation of a method described in Harlow and Lane (1988). The tissue in NP40 lysis buffer was mechanically disrupted, sonicated and the protein containing supernatant collected by ultracentrifugation at 10,000 G for 10 minutes. The concentration obtained was estimated using a commercially available colorimetric assay according to the manufacturers instructions (BioRad UK) based on the method described by originally described by Bradford (1976).

2.2.4 Collection and Processing of Plasma Samples.

Heparinised venous blood samples were obtained from a total of 22 patients with CFA, 22 patients with sarcoidosis from 33 healthy volunteers within the Pathology Department. None of the patients were on specific therapy at the time of sampling and all the volunteers were well and on no medication (see Appendix A for further details).

2.2.3 The plasma fraction was obtained by centrifugation of the blood sample at 1000 G for 20 minutes on Ficoll-Hypaque (Nycomed, Norway). The plasma was removed, aliquoted and stored at -70°C.

2.2.4.1 Purification of IgG from Plasma Samples.

IgG was obtained from human plasma samples by immunoaffinity purification using Protein G coated Sepharose beads (Pharmacia) using the method described in Harlow and Lane (1988). Briefly plasma diluted 1:2 with PBS was passed over a protein G column and allowed to adsorb at room temperature under 1 G. Non-adsorbed material was washed off with 5 column volumes of PBS. Bound IgG was eluted with 1 column volume of 0.1M glycine at pH 2.5 at room temperature. The eluate was restored immediately to neutral pH by the addition of 1M tris/HCl pH 7.2. The IgG fraction was then aliquoted and frozen at -70°C until use.

2.2.4.2 Biotinylation of Purified IgG.

Biotinylation was performed based on methods described by Pohlit *et al* (1979). Purified IgG was dialysed against 0.1M NaHCO₃ (pH 8.2-8.6) over 24 hours with 3 changes of buffer. The IgG was then recovered, the concentration measured as described in 2.2.3.1 and standardised to give 1mg of IgG in 1ml of buffer.

A fresh solution of biotin succinamide ester (N-hydroxy-succinimido-biotin, Pierce & Warriner USA) at a concentration of 1mg/ml in dimethylsulphoxide (DMSO, Sigma UK) was incubated with the IgG at room temperature for 4 hours. This was then dialysed overnight against TBS with 0.2% sodium azide, aliquoted and stored at -70°C.

2.2.5 Collection and Processing of Bronchoalveolar Lavage Samples.

20 ml aliquots of bronchoalveolar lavage (BAL) fluid were obtained from 24 patients undergoing bronchoscopy at the Western General Hospital, Edinburgh for the investigation of diffuse lung disease.

2.2.5.1 Preparation of Cytospins.

The samples were centrifuged and the BAL fluid aliquoted and stored at -70°C. The cellular component of the lavage sample was used to produce cytopsin preparations which were acetone fixed and stored at -20°C.

2.2.5.2 Purification of IgG.

IgG was purified from BAL fluid using Protein G affinity columns as described in 2.2.4.1.

2.2.5.3. Concentration of BAL Protein for SDS-PAGE.

The protein content of BAL fluid was concentrated by placing samples in Viskose Dialysis Tubing (Fisons UK) and covering them with polyethylene glycol crystals (Fisons UK). The samples were left at 4°C overnight and the concentrated protein recovered.

2.3. Cell Culture.

A type II alveolar epithelial cell line, A549, (Lieber *et al* 1976) was kindly donated by Dr W. McNee, Department of Medicine, Edinburgh University as was the L2P6 pulmonary fibroblast cell line. Other cell lines used were available within the laboratory and were kindly donated by Dr. Sarah EM Howie (Jurkat and H9).

2.3.1 Culture Conditions.

All cells were maintained in RPMI medium supplemented with glutamine 0.4mM/ml, penicillin 100U/ml and streptomycin 100µg/ml and 10% foetal calf serum (all Gibco BRL UK). Cells grown in monolayers were detached using a non-enzymatic cell releasing buffer (Gibco BRL UK) and were never exposed to trypsin.

2.3.1.1 Preparation of Cytospins.

Cells detached as described above were washed in PBS by centrifugation at 300G for 5 minutes and used to produce cytopins at a cell density of 1×10^5 cells per slide. Slides were then fixed in acetone for 10 minutes at room temperature and stored at -20°C.

2.3.2 Protein Extraction from Cell Lines.

2.3.2.1 NP-40 Protein Extraction.

Protein extracts were prepared from cell pellets in a manner similar to that described for biopsy tissue (2.2.3.1). Cell pellets were, however, not subjected to mechanical disruption prior to sonication.

2.3.2.2 Differential Preparation of Microsomal and Cytoplasmic Proteins.

Differential extraction of microsomal and cytoplasmic proteins was performed using ultracentrifugation as described by Meehan *et al* (1988). A cell pellet was disrupted by repeat freeze/thaw cycles in a KCl-phosphate buffer followed by centrifugation at 10,000 G for 20 minutes to remove the cell membranes. The supernatant was ultracentrifuged at 90,000 G for 80 minutes and the supernatant saved as the cytosolic protein fraction. The pellet was resuspended in KCl-phosphate buffer and centrifuged at 90,000 G for a further 1 hour and the supernatant discarded. The pellet was resuspended in 1ml PBS and retained as the microsomal fraction. The protein yield was estimated for each sample (section 2.2.3.1) which was then aliquoted and stored at -70°C.

2.3.3 MTT Assay.

The MTT assay was performed using the method described by Mossman (1983). Cells were cultured in tissue culture grade flat-bottomed 96 well microtitre plates (Costar USA) as described above. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma UK) was added to each well to give a final concentration of 1mg/ml. The plates were then incubated at 37°C for 4 hours. Acidified anhydrous isopropanol (Sigma UK) was added (100µl/well) and the precipitate dissolved by mixing. The plates were then read using an automatic plate reader (Dynatech 5000) at 570nm.

2.4 Polyacrylamide Gel Electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) was performed according to the methods described by Harlow and Lane (1988) and using the buffers described by the manufacturers of the Protean II Mini-System (Biorad Ltd., UK). 7.5% polyacrylamide running gels were cast and overlaid with 4% stacking gels. Protein samples obtained as described above were placed in 100µl of sample buffer, boiled for 3 minutes and run under reducing conditions at a constant voltage. Biotinylated molecular weight standards (BioRad Ltd. UK; 200,000 - 45,000 kDa) were run on all gels in a reference track alongside the test sample. The proteins were electrophoretically transferred to nitrocellulose (Amersham, Hybond-C, 0.45µM) sheets which were dried and stored at 4°C until required.

2.5. Western Blotting.

Western blotting was performed with plasma, serum and immunoglobulin samples either in glass staining dishes or a specialised clamp (BioRad UK Ltd) which divided the nitrocellulose membrane into 20 different tracks allowing the simultaneous testing of multiple reagents on a single nitrocellulose sheet.

A variety of detection systems were employed depending on the nature of the primary reagent used for the blot. All utilised incubation of the nitrocellulose sheet with a series of monoclonal and/or polyclonal antibodies (for details see Appendix B) designed to detect specific interactions. Visualisation of reactive bands was performed using the alkaline phosphatase substrate 5-bromo-4-chloro 3 indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) (both Sigma UK).

Following blotting a standard curve was produced for each experiment from the molecular weight markers. This was calculated by graphing the \log_{10} of the molecular weight of each standard against the distance run in millimetres expressed as a percentage of the distance covered by the gel front. From this graph the molecular weights of bands detected by the plasma samples on the blots could be calculated.

2.6. Production of Xeno-Antibodies.

A female Dutch White rabbit was immunised with partially purified antigen obtained using 2 separate techniques from CFA lung biopsies.

2.6.1 Affinity Purification of Antigen from Lung Tissue.

Affinity purification columns were produced as described in Harlow and Lane (1988). Cyanogen bromide activated Sepharose beads (Sigma UK) were coupled to IgG purified from CFA patient plasma as described in section 2.2.4.1. The IgG coated beads were incubated with protein extracts derived from CFA lung biopsy tissue as described in section 2.3.1, washed and adherent antigen eluted under acid conditions with 0.1 M glycine. The eluent was absorbed with Protein G conjugated Sepharose beads (Pharmacia) to adsorb any IgG stripped from the column with the recovered antigen. Dot blots were then performed with pooled plasma samples from CFA patients (not those used to construct the column) and controls in order to determine whether any antigen had been obtained. The blots were

developed using the same detection system as was used for Western blotting.

2.6.1 Partial Purification by SDS-PAGE.

Pooled protein extracts from open lung biopsies from patients with CFA were separated by SDS-PAGE and transferred to nitrocellulose sheets as described above (section 2.4). The position of the 70-90 kDa protein(s) on the membrane was identified by blotting one edge of the blot which had been cut from the nitrocellulose sheet. The strip containing the protein(s) of this molecular weight in the remainder of the nitrocellulose sheet was then excised, solubilised in DMSO at 37°C and reprecipitated with phosphate buffered saline (PBS) to give a fine particulate suspension of protein(s) bound to nitrocellulose (Knudson 1985).

2.6.3 Immunisation Schedule.

Antigen obtained from both sources was pooled and mixed 1:1 with alum (Pierce and Warriner, UK Ltd) prior to storage at -70°C. Following the collection of baseline serum samples from the rabbit 3 immunisations were performed at 4 weekly intervals using the antigenic material described above. 4 weeks following the final dose a final immune sample of serum was taken.

2.6.4 Preparation of Immune and Control IgG.

IgG was purified from both the control and immune samples using Protein A conjugated Sepharose beads (Pharmacia) as described in section 2.2.4.1. The purified IgG was then adsorbed with human IgG coated agarose beads (Sigma UK) to remove any anti-human IgG reactivity. The protein concentration was then estimated using the method previously described (2.2.3.1) and standardised to 1mg/ml. The samples were then aliquoted and stored at -70°C.

2.7. Immunoprecipitation.

Immunoprecipitation of antigen from CFA lung biopsy protein extracts was performed as described in Harlow and Lane (1988). 6 mg of protein in 3 mls of NP 40 lysis buffer extracted from 3 open lung biopsy specimens was obtained. The protein was precleared by incubating the material with 25µl of the control rabbit IgG on ice for 30 minutes. Complexes formed were

removed by incubation with 100 μ l 10% Protein A conjugated Sepharose beads in PBS (Pharmacia) at 4°C for 30 minutes followed by centrifugation (10,000 G for 5 minutes). Immune rabbit IgG was then added to the protein mixture to give a final IgG concentration of 100 μ g/ml (ie a 1:10 dilution) incubated for 4 hours on ice and recovered as above using Protein A conjugated Sepharose beads.

The beads were washed in decreasing salt buffers and then boiled in the presence of sodium lauryl sulphate (SDS, Sigma UK) and dithiothreitol in order to disassociate the beads, IgG and protein. The beads were recovered by centrifugation (10,000 G for 5 minutes) and the supernatant run on 7.5% polyacrylamide gels as described in section 2.4. The gels were then either stained with Coomassie Blue (Sigma UK) in order to identify the position and molecular weights of the precipitated proteins or transferred to nitrocellulose for subsequent Western blotting.

2.8. Immunohistochemistry.

Antibody reagents unless otherwise stated were obtained from Dako UK.

2.8.1 Antigen Retrieval Pre-treatment of Sections Prior to Immunohistochemistry.

Two methods were used to pre-treat formalin fixed, paraffin embedded sections prior to immunohistochemical staining where required.

2.8.1.1 Trypsin Digestion.

Sections were incubated with 0.1% trypsin (ICN Biomedical Inc. USA) in 0.1% calcium chloride (pH 7.8) for 15 minutes at 37°C.

2.8.1.2 Microwaving.

Using the method described by Cattoretti *et al* (1992) sections were heated in 0.01M citric acid (pH 9.0) for 15 minutes. The sections were then allowed to cool in the buffer.

2.8.2 Immunofluorescence.

Immunofluorescent immunohistochemistry was performed on cryostat sections of tissue obtained and processed as described in 2.2.2. Sections were incubated with the primary antibody, washed and then incubated with

an appropriate fluorescein isothiocyanate (FITC) conjugated secondary antibody. The sections were then mounted with PBS/glycerine.

2.8.3 Immunoperoxidase.

Formalin fixed, paraffin embedded or cryostat sections were incubated with primary antibody, washed with TBS, incubated with an appropriate biotin conjugated secondary antibody, washed again, incubated with horseradish peroxidase conjugated avidin (Vector Labs) and visualised with the peroxidase substrate diaminobenzidine (DAB, Sigma UK). Sections were counterstained with haematoxylin, dehydrated and mounted.

Endogenous peroxidase in tissues was blocked by incubation with 1% H_2O_2 in water at 37°C for 10 minutes prior to staining.

2.8.4 Immuno-alkaline phosphatase.

Two alkaline phosphatase staining methods were used.

2.8.4.1 ABC Method.

Tissue sections or cytospin preparations were stained as described in section 2.9.2 with the substitution of an alkaline phosphatase avidin biotin complex (ABC) in place of the peroxidase conjugate. Visualisation was performed with the alkaline phosphatase substrate Vector Red (Vector Labs UK) with the inclusion of 1mM levamisole in the buffer to block endogenous enzyme activity.

2.8.4.2 Alkaline Phosphatase-Protein A Conjugate Method.

Cryostat sections or cytospin preparations were incubated with Protein A (Sigma UK), washed and further incubated with neat fresh human plasma. Following further washes the sections were incubated with the primary antibody followed by an alkaline phosphatase conjugated Protein A complex (Sigma UK). Visualisation was performed as described in section 2.9.3.1.

2.9. Flow Cytometry.

2.9.1 Preparation of Cells for Flow Cytometry.

Cells were obtained from culture as described in section 2.3 and were stained using an indirect immunofluorescent technique in suspension similar to that described in section 2.9.2. Cells were either stained in the fresh state for surface markers or after permeabilisation for intra-cellular markers.

Permeabilisation was performed by fixing the cells in 2% paraformaldehyde in PBS for 15 minutes at 4°C, washing in PBS followed by incubation with 0.1% Triton X for 5 minutes. The cells were then again washed in PBS prior to staining.

2.9.2 Flow Cytometry.

Flow cytometry was performed using a EPICS CS System (Coulter Corporation USA) gated to detect viable cells by their forward and side light scatter pattern. Results were expressed as the percentage of the total cells counted that showed positive staining.

2.10. Enzyme Linked Immunosorbance Assay (ELISA).

2.10.1 Sandwich ELISA.

Serial dilutions of plasma samples were coated in triplicate onto ELISA grade 96 well plates at 100µl/well (Costar UK) using a carbonate/bicarbonate buffer pH 9.6 (Sigma UK). The wells were then blocked for 10 minutes with 100µl/well 1% BSA in TBS and washed x 3 with washing buffer (PBS containing 0.01% BSA and 0.05% Tween-20). The plates were then incubated with 100µl/well of an anti-human IgG monoclonal antibody (Sigma UK), washed as before and detected with 100ml/well of a peroxidase conjugated rabbit anti-mouse monoclonal antibody (Sigma UK). The plates were then developed with the peroxidase substrate o-phenylenediamine dihydrochloride (Sigma UK), the reaction stopped with 25 µl/well 3N HCl and the plate read using an automatic plate reader (Dynatech 5000) using a 490nm test filter and a 620nm reference filter.

2.10.2 Antigen Capture ELISA.

Immune rabbit IgG, optimally diluted in carbonate/bicarbonate buffer (Sigma UK), was coated onto 96 well plates as above. The plates were blocked with 1% BSA, washed, incubated with 50µg/well protein extracts produced from A549 cells and washed again. A further incubation with either control or CFA plasma, serially diluted in triplicate wells, was then performed. Detection was achieved using an appropriate peroxidase conjugated monoclonal antibody directed against the human IgG. The plates were then developed and read as described in 2.11.1.

2.11. Statistical Analyses.

All analyses were performed either using a Chi-Square or Mann-Whitney Test (Minitab Statistics package, Minitab Inc. PA. USA) .

SCREENING OF PLASMA SAMPLES FOR AUTOANTIBODIES TO LUNG ANTIGENS.

In CFA there is evidence of a persistent immune reaction in the pulmonary interstitium with resulting fibrosis. The mechanisms which drive these chronic responses are unknown but possible immunoglobulin production locally within the lung and immune complex deposition has been previously demonstrated in patients with CFA (see section 1.10). Attempts to identify circulating autoantibodies using immunofluorescent techniques with patient serum and cryostat sections of lung tissue have, however, failed to provide conclusive results (Turner-Warwick and Doniach 1965, Ehringer 1991). This is principally because the lung contains large amounts of both free and cell bound immunoglobulin to which labelled anti-human immunoglobulin sticks resulting in extensive non-specific staining. The technique of Western blotting circumvents this problem as the proteins present in the lung, including immunoglobulins, are separated according to molecular weight (Harlow and Lane 1988). This allows identification of specific reactions between antibodies in plasma with non-immunoglobulin antigenic proteins.

The experiments reported in this chapter were designed to determine if autoantibodies to lung protein(s) could be detected in the plasma of patients with CFA using Western blotting.

3.1 Detection of IgG Autoantibodies.

Plasma samples were obtained as described in section 2.2.4 from 22 patients with CFA (14M:8F, mean age(range):66.2(49-80)). 19 of whom had undergone open or thoracoscopic lung biopsy, 22 patients (13M:9F, mean age(range):43.5(28-63) with biopsy proven pulmonary sarcoidosis (17 by transbronchial biopsy and 5 by open or thoracoscopic lung biopsy) and 33 healthy volunteers of whom 17 were non-smokers (10M:7F, mean age(range): 41.5(28-58) and 16 smokers (11M:5F, mean age(range):40.3(22-59)). For further details see appendix A.

Aliquots of the plasma samples were used in Western blotting experiments (section 2.5) with protein extracts derived from pulmonary and non-pulmonary tissues (section 2.2.3) separated on 7.5% SDS polyacrylamide gels (section 2.4). The interaction of IgG in the plasma samples with protein on the nitrocellulose sheets was detected using a monoclonal anti-human IgG γ and the alkaline phosphatase detection system

described in section 2.5. The molecular weights of reactive bands on the blots were then calculated for each sample from the standards as described in section 2.5.

3.1.1 CFA Protein Extracts.

CFA and control plasma samples were blotted against protein derived from CFA biopsy material to identify autoantibodies recognising lung antigen(s) that were specific to the CFA samples (representative blots are shown in Figs. 3.1 and 3.2). The results revealed the presence of bands extending across the blots due to non-specific reactivity of the detection system with immunoglobulin and biotin which was present in the original lung tissue. In addition many individuals in both groups showed evidence of antibodies reacting with a variety of proteins of differing molecular weights. Only bands in the 70-90 kDa range were found to have a significant correlation with the CFA samples with the others arising in both patient and control groups. Serial dilution of the plasma samples resulted in progressive loss of reactivity between the samples and the protein on the nitrocellulose (Fig. 3.3) at this molecular weight confirming that the bands were the result of antibody in the plasma sample and not an artefact of the detection system.

The results obtained blotting the sera against 3 different CFA protein samples are indicated in Table 3.1. No statistical difference was observed in the frequency with which the 70-90 kDa antigen was detected in the 3 different extracts (9/9, 12/14, 18/22) by the CFA patient plasma. The control plasma samples in comparison showed a significantly lower frequency of positivity to the antigen in this weight range (non-smoking control plasma samples 1/17; smoking control plasma samples 0/16, $p < 0.001$, Chi Square Test).

20 of the 22 plasma samples (91%) were blotted on 2 or more of the CFA protein extracts. Of these 20 plasma samples 14 (70%) showed 100% concordance of positivity on the protein samples that they were tested against. The remaining 6 plasma samples were positive on at least one protein extract. Further evidence that the antigen detected was generally present in CFA biopsies was obtained in experiments in which 4 CFA protein extracts were run in parallel and blotted separately with pooled CFA and control plasma samples. CFA, but not the control plasma, detected the antigen in all 4 samples confirming that the presence of the antigen appeared to be a feature of CFA lung tissue.

3.1.1 In order to examine if autoantibodies to these protein(s) were specific to the CFA plasma samples, or represented a non-specific response in patients to the presence of inflammation in the pulmonary interstitium, 18 sarcoid plasma samples were blotted against CFA protein extracts. Only 5/18 of the sarcoid plasma samples contained autoantibodies which recognised protein(s) in the 70-90 kDa range (representative data shown in Fig 3.4). This was significantly fewer than for the CFA plasma samples ($p < 0.001$, Chi Square Test) but not significantly different from the control samples indicating that generation of the apparent humoral response to the antigen was not a non-specific feature of local inflammation in the lung.

3.1.2 Sarcoid Protein Extract

As CFA plasma samples contained a high frequency of autoantibodies to 70-90 kDa lung protein(s) present in CFA lung tissue it was of interest to determine whether the same protein(s) were present in lung tissue from other patients. The same CFA plasma samples were, therefore, blotted onto protein extracts derived from sarcoid lung biopsies. 18/19 CFA plasma samples were shown to react with 70-90 kDa protein(s) compared with only 6/22 sarcoid plasma samples ($p < 0.001$) (representative data Fig. 3.5). This frequency of positivity on the sarcoid extract was not significantly different from that obtained using the CFA extract for either group (Chi Square Test). 5/17 of the non-smoking control plasma samples were positive on the 70-90 kDa region on the sarcoid extract and this was found to be significantly more frequent than on the CFA extract ($p < 0.05$ Chi Square Test).

3.1.3 Control Lung Extracts.

In order to determine if the protein(s) detected by the autoantibodies in the CFA plasma were normal lung constituents or only present in patients with inflammatory lung disease CFA plasma samples were blotted against protein extracts from control lung tissue. 12/15 of the CFA plasma samples were positive, recognising 70-90 kDa protein(s) in the extract (Fig. 3.6). The bands obtained were very much fainter than with the ILD extracts but were still present at a frequency not significantly different from that obtained on the CFA or sarcoid protein extracts.

3.1.4 Overall Incidence of IgG Autoantibody to the 70-90 kDa Pulmonary Antigen.

Examination of the data presented in Table 3.1 indicates that 20/22 of the CFA plasma samples were blotted against either 3 or 4 different lung protein extracts (2 were only blotted against 1 extract). The percentage of protein extracts on which each plasma sample had been tested and found to detect the antigen was calculated. Only 1 of the 20 plasma samples detected the antigen in less than 50% of the pulmonary protein extracts against which it was blotted (number 13, 1/4) indicating possible absence of the autoantibody in this single case. Analysis of the data in this manner indicated an incidence of the IgG autoantibody of 95% (19/20) in the CFA plasma samples tested.

3.1.5 Non-pulmonary Tissue Extracts.

In order to ascertain if the proteins detected by the autoantibodies were lung specific Western blotting was performed with protein derived from a variety of non-pulmonary tissues. CFA plasma samples were blotted against extracts of colonic mucosa, tonsil, liver and skin (representative data Fig. 3.7) which were produced in a manner identical to that for the lung tissues (section 2.2.3). The frequency of positive bands in the 70-90 kDa range obtained (normal colonic mucosa 3/13; liver 3/14; tonsil 3/14; skin 4/14) was significantly lower than on the control lung extract ($p<0.05$ Chi Square Test) and ILD lung extracts ($p<0.001$ Chi Square Test).

Table 3.2 indicates the results obtained for each plasma sample in detail. 8 out of the 13 bands identified were attributable to only 3 of the plasma samples (numbers 3, 4 and 13). This indicated that the majority of CFA plasma samples that contained the autoantibody to the lung protein failed to recognise antigen of similar molecular weight in non-pulmonary tissues. Interestingly, however, case number 13 was positive in this molecular weight range against the 3 non-pulmonary tissues that it was tested against. This was the same case as was found to be positive on only 1 of 4 pulmonary samples and suggests that this individual may be showing a different pattern of immune response compared to the others tested..

A summary of the results obtained screening for the presence of IgG autoantibodies to the 70-90 kDa antigen is shown in Table 3.3.

3.2 Relative IgG Concentration of Control, CFA and Sarcoid Plasma Samples.

In order to exclude the possibility that the increased frequency of IgG reactivity with the 70-90 kDa antigen on the nitrocellulose was an artefact due to hypergammaglobulinaemia in the CFA plasma samples a sandwich ELISA (2.10.1) was performed to compare the relative IgG content of the different samples. The mean OD value of triplicate wells for serial dilutions of each plasma sample used in the above experiments was calculated. The results of the 1:10,000 dilution were used for analysis as they were found to be on the linear portion of the dilution curve. Statistical analyses were performed using a Mann-Whitney Test.

Comparison of the OD values obtained at this dilution for the 3 groups (Fig. 3.8) [median (range) Controls 1.158(1.000-1.501), CFA 1.403(1.123-1.825), Sarcoid 1.707(1.455-1.866)] confirmed a significantly higher IgG content in the sarcoid ($p < 0.001$) and CFA ($p < 0.001$) plasma compared with the control samples although considerable overlap was apparent. The IgG content of the sarcoid samples were also shown to be significantly higher than the CFA samples ($p < 0.001$) indicating that the blotting results could not simply be due to the relative IgG content of the test plasma samples. In addition the CFA case that appeared not to contain the autoantibody (number 13) was not significantly different in its relative IgG concentration (OD = 1.355) compared with the CFA group as a whole [median(range): 1.403(1.123-1.825)].

3.3 Detection of IgM and IgA Autoantibodies in CFA Plasma.

Western blotting experiments were performed to detect IgM and IgA autoantibodies against CFA lung proteins of similar molecular weight by substitution of a monoclonal anti-human IgM μ (DA126, kindly donated by Dr. Keith Guy) or anti-human IgA α (Sigma UK) in place of the anti-human IgG in the detection system. These experiments revealed no evidence of specific IgM or IgA (Fig. 3.9) autoantibodies directed against CFA lung protein(s) in this weight range.

3.4 Comment.

Previous attempts to demonstrate autoantibodies to pulmonary antigens in CFA have been inconclusive due to a lack of specificity in the techniques employed (Turner-Warwick and Doniach 1965, Ehringer 1991).

SDS-PAGE and Western blotting circumvents this problem by separating the individual lung proteins, including immunoglobulins, on the basis of molecular weight thus allowing identification of specific interactions between autoantibodies and lung proteins to be observed. The reproducibility of this technique appears good with a 70% absolute concordance rate in autoantibody detection against 3 separate CFA lung biopsy specimens. In addition the results suggest that detection of the autoantibody has an overall sensitivity of 95% (19/20) and a specificity of 76% (19/25) for the diagnosis of CFA.

The mean age of the CFA patients was significantly higher than that of the sarcoid patients ($p < 0.01$, Mann-Whitney) reflecting the populations that these conditions affect. A similar difference was also present between the CFA patients and the healthy control group. This arose as it was considered necessary to use healthy controls who were not on any medication and who were not patients in order to avoid possible confounding factors introduced by other disease processes. Control samples were thus obtained from members of the Pathology Department and this introduced a bias to a younger age group. The age range of the controls and the CFA patients, however, overlapped and there was no suggestion that age affected the probability of positivity in either group.

Calculation of the molecular weight of the antigen detected by each sample in each experiment revealed some variation within the 70-90kDa range. This appeared to be due to a degree of error in estimating the molecular weight of the bands. This was perhaps not surprising as the calculation depended on measuring the position of each band relative to the top of the gel with a ruler for each track. This error was estimated to be in the order of 10-15% using a monoclonal antibody to vimentin (56 kDa).

Analysis of the results combining the CFA, sarcoid and control lung extracts suggested that only one of 20 cases was unlikely to contain the autoantibody. In addition this case was the one apparently reacting with antigen of 70-90 kDa in the non-pulmonary tissues. Review of the biopsy from this individual confirmed that he had CFA and did not suggest any particular difference from other cases. It may be possible that this individual merely had a low titre of the autoantibody rather than being completely negative as it did blot positively on one of the CFA samples. The presence or absence of a band on Western blotting is dependent on the concentration of antibody and antigen. While the total protein loaded on the gel can be

standardised the relative contribution of the antigen of interest may be higher in some samples than others. Thus a sample from a patient with a low titre may only score positively on some blots and negatively on others. Similarly the intensity of the bands obtained on any one blot will reflect the titre of antibody applied and examination of the results (Fig. 3.1) certainly indicated the presence a wide variation in the intensity of bands obtained with different plasma samples. This implies that CFA patients have a range of autoantibody titres to this antigen although the significance of this is unclear.

The presence of the autoantibodies in 95% of CFA patient plasma samples examined in this study is higher than the proportion of cases than for which immune complex deposition (Nagaya *et al* 1969 and 1973, Turner-Warwick *et al* 1971, Hogan *et al* 1978, Schwartz *et al* 1978, Dreisen *et al* 1978, Pratt *et al* 1979) or circulating immune complexes have been detected (Dreisen *et al* 1978, Turner-Warwick 1978, Pratt *et al* 1979, Haslam *et al* 1978 and 1982, Crystal *et al* 1984). This may, however, be a reflection of the sampling used in other studies. It has been suggested that the presence of detectable immune complexes in CFA lung biopsies is correlated with areas of active disease and that they cannot be detected in end stage disease (Nagaya *et al* 1969, Dreisen *et al* 1978, Turner-Warwick *et al* 1971). As a variable proportion of biopsies from patients may show only inactive disease it is therefore not surprising that immune complexes are detectable in some cases but not others.

In addition the presence of detectable circulating immune complexes may be influenced by the titre of the autoantibody forming the complex. The autoantibody detected in this study certainly appeared to have a variable titre between patients although no correlation between the presence of circulating complexes and any measure of titre was attempted. Thus previous data showing a lower and more variable incidence of circulating and lung immune complexes, therefore, need not be in contradiction of the almost uniform presence of an autoantibody in these patients.

The presence of IgG and not IgM autoantibodies may reflect the fact that the disease process is well established by the time the samples were obtained and that an earlier IgM response has waned. This also appears compatible with the observation that the majority of immune complexes detectable in CFA are of the IgG type (Nagaya *et al* 1973, Dreisen *et al*

1978, Dall'Aglio *et al* 1988) although IgM and IgA have been described (Nagaya *et al* 1969, Pratt *et al* 1979).

The results with the sarcoid sera indicated that the presence of the IgG autoantibodies was not simply a non-specific reaction to interstitial inflammation as the frequency of the antibody in these individuals was not significantly higher than in the healthy controls. This suggested that the presence of the autoantibody in the CFA patients might therefore not simply be a reflection of a local inflammatory process but might have significance in the pathogenesis of the disease. While these experiments were unable to provide data on the nature and location of the antigen it seems likely to be endogenous with possible upregulation in the inflamed lung samples. The data obtained with the non-pulmonary tissues suggests that the antigen may be lung specific as the majority of CFA plasma samples did not detect antigen on these blots. Most (8/13) of the bands were accounted for by three patients raising the possibility that these might in some way represent a subgroup.

The results obtained in these experiments have for the first time successfully demonstrated the presence of IgG autoantibodies to an extractable lung antigen in patients with CFA. While these techniques gave no clue to the identity of the antigen they suggest that it may be endogenous and lung specific.

3.5 Conclusion.

Patients with CFA have a high frequency of circulating IgG autoantibodies which recognise pulmonary protein(s) of 70-90 kDa. Such antibodies are not detectable in patients with sarcoid significantly more frequently than healthy controls. The antigenic protein appears to be present in control lung tissue but may be more abundant in ILD lung biopsy tissue.

Table 3.1

Analysis of the results obtained screening CFA plasma samples for IgG autoantibodies to the 70-90 kDa antigen on pulmonary tissue. The results obtained for each plasma sample blotted against the different sources of lung protein is indicated. The proportion of cases blotting positively with each source of protein is indicated as are the percentage of the protein extracts in which each plasma sample detected the antigen.

X = band present in the 70-90 kDa molecular weight range.

O = no band detected in the 70-90 kDa molecular weight range.

ND = not done.

Table 3.1

Serum Sample No.	CFA Protein Sample 1.	CFA Protein Sample 2.	CFA Protein Sample 3	Sarcoid Protein Sample.	Control Protein Sample.	% of Protein Samples Tested in Which Antigen was Detected
1	ND	X	O	X	X	75
2	X	ND	X	X	X	100
3	X	ND	O	X	X	75
4	X	ND	X	X	X	100
5	X	ND	X	X	O	75
6	X	ND	O	X	X	75
7	ND	X	X	X	X	100
8	ND	O	X	X	X	75
9	ND	X	X	X	X	100
10	ND	O	X	X	O	50
11	ND	X	X	X	ND	100
12	ND	X	X	X	ND	100
13	ND	X	O	O	O	25
14	ND	X	X	ND	X	100
15	ND	X	X	X	X	100
16	ND	X	X	X	X	100
17	X	X	X	X	ND	100
18	X	X	X	X	ND	100
19	X	X	X	X	ND	100
20	X	ND	X	X	X	100
21	ND	ND	X	ND	ND	(100)
22	ND	ND	X	ND	ND	(100)
Proportion of Cases Positive	9/9	12/14	18/22	18/19	12/15	

Table 3.2.

Analysis of the results obtained screening CFA plasma samples for IgG autoantibodies to the 70-90 kDa antigen on non-pulmonary tissues. The results obtained for each plasma sample blotted against different tissues is shown. The proportion of cases blotting positively is indicated for each protein source as are the percentage of the protein extracts in which each plasma sample detected the antigen.

X = band present in the 70-90 kDa molecular weight range.

O = no band detected in the 70-90 kDa molecular weight range.

ND = not done.

Table 3.2.

Plasma Sample No.	Colonic Mucosal Protein Extract	Liver Protein Extract	Skin Protein Extract	Tonsil Protein Extract	% of Protein Samples Tested in Which Antigen was Detected
1	O	O	O	O	0
2	O	O	X	O	25
3	X	X	O	X	75
4	O	X	X	O	50
5	O	O	O	X	25
6	X	O	O	O	0
7	O	O	O	O	0
8	O	O	O	O	0
9	O	O	O	O	0
10	X	O	O	O	25
11	O	O	O	O	0
12	O	O	O	O	0
13	ND	X	X	X	100
14	O	O	X	O	25
Proportion of Cases Positive	3/13	3/14	4/14	3/14	

Table 3.3.

Summary of results obtained screening CFA plasma samples for the presence of IgG autoantibodies to the 70-90 kDa antigen using pulmonary and non-pulmonary tissues.

* The frequency of positive bands in the 70-90 kDa range is significantly more frequent ($p < 0.001$) with the CFA plasma samples than the sarcoid or control plasma samples when blotted on CFA or sarcoid lung biopsy protein extracts.

\$ The frequency of positive bands in the 70-90 kDa range with CFA plasma samples on control, non-ILD, lung tissue is not significantly different from that with CFA or sarcoid lung tissue.

The frequency of positive bands in the 70-90 kDa range with CFA plasma using non-pulmonary tissues is significantly less frequent than on the control lung extract ($p < 0.05$) or the ILD extracts ($p < 0.001$).

Table 3.3.

Tissue source	Frequency of Plasma Samples Recognising a 70-90kDa Protein in Tissue Extracts.			
	CFA Plasma	Sarcoid Plasma	Non-Smoking Control Plasma	Smoking Control Plasma
CFA lung biopsy 3	18/22 (82%)*	5/18 (28%)	1/17 (6%)	0/16 (0%)
CFA lung biopsy 2	12/14 (86%)*			
CFA lung biopsy 1	9/9 (100%)*			
Sarcoid lung biopsy	18/19 (95%)*	6/22 (27%)	5/17 (23%)	
Control lung	12/15 (80%)*			
Colonic mucosa	3/13 (23%)*			
Liver	3/14 (21%)*			
Tonsil	3/14 (21%)*			
Skin	4/14 (29%)*			

Figure 3.1

Illustration of a composite Western blot showing the results obtained blotting 22 CFA plasma samples on a protein extract produced from CFA lung biopsy material using a monoclonal anti-human IgG γ as described in section 2.5. The molecular weight markers are indicated in kDa on the right. Positive bands of varying intensity are identified in the 70-90 kDa range (arrow) for 18/22 plasma samples (lanes 2, 4, 5, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, 20, 21 and 22). The biopsy material in the blot shown corresponds to the plasma shown in lane 22.

Figure 3.2

Illustration of a Western blot showing the results obtained blotting 15 non-smoking control plasma samples on a protein extract produced from CFA lung biopsy material and developed as described in section 2.5 using a monoclonal anti-human IgG γ . The molecular weight markers are indicated in kDa on the right. Only 1 sample (lane 13) shows positivity in the 70-90kDa range (arrow). A similar low frequency of positivity was obtained with smoking controls (0/16).

Figure 3.1

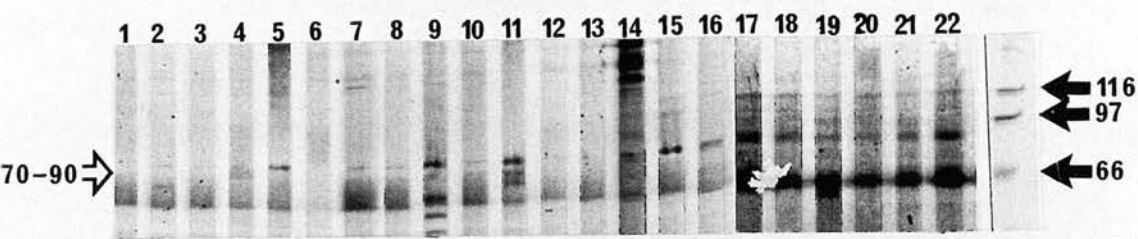
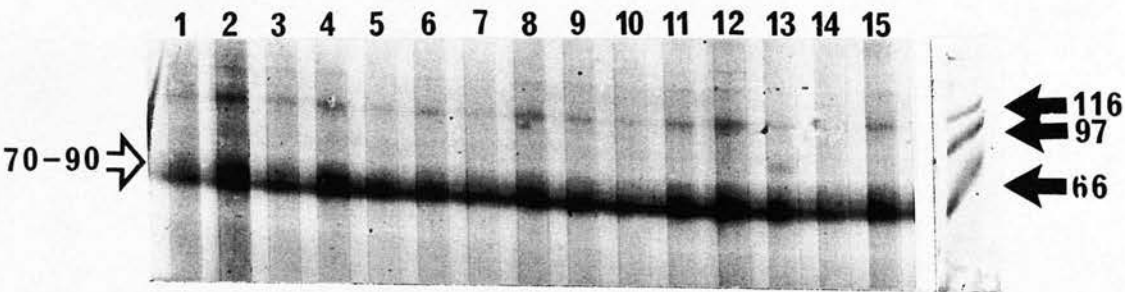


Figure 3.2



84a

Figure 3.3

Western blot developed using a monoclonal anti-human IgG γ as described in section 2.5 showing progressive loss of the positive band in the 70-90 kDa range with serial dilution of the plasma sample (arrow). No band is detected in this weight range with the control sample. These results confirm that the band detected is due to antibody in the patient plasma interacting with protein on the nitrocellulose sample rather than an artefact of the detection system.

Figure 3.4

Illustration of representative Western blot results obtained blotting 17 sarcoid plasma samples on a protein extract produced from CFA lung biopsy material using a monoclonal anti-human IgG γ as described in section 2.5. The molecular weight markers are indicated in kDa on the right. Bands of varying intensity were detected in the 70-90 kDa region (arrow) with 5 of the samples (lanes 3,4,9,14 and 16).

Figure 3.3

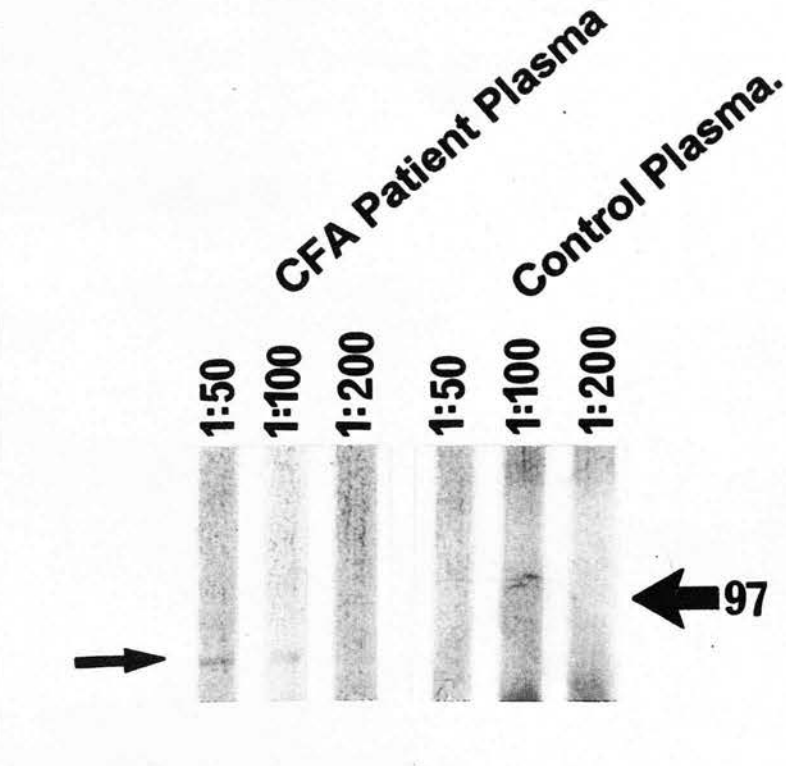


Figure 3.4

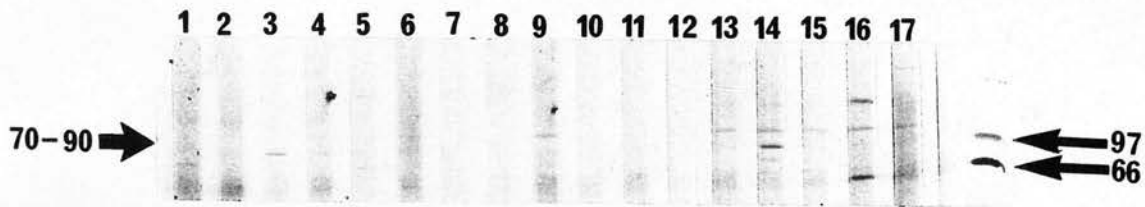


Figure 3.5

Illustration of a Western blot showing representative results obtained blotting CFA (lanes 1-6) and sarcoid (lanes 7-13) plasma samples on a protein extract produced from sarcoid lung tissue and using a monoclonal anti-human IgG γ as described in section 2.5. The molecular weight markers are indicated in kDa on the right. 5/6 of the CFA plasma samples illustrated are positive in the 70-90 kDa range (arrow) (lanes 1, 2, 3, 4, and 6) compared with only 1/7 of the sarcoid plasma samples (lane 10).

Figure 3.6

Illustration of a Western blot showing the results obtained blotting 15 CFA plasma samples on a protein extract produced from control, non-ILD, lung tissue using a monoclonal anti-human IgG γ as described in section 2.5. The molecular weight markers are indicated in kDa on the right. 12/15 of the plasma samples showed positivity in the 70-90 kDa range (arrow) (lanes 1, 2, 3, 4, 5, 7, 8, 10, 11, 13, 14, and 15). The positive bands were, however, very much fainter than in figure 3.1 despite employing a longer development period, as indicated by the more intense staining of the non-specific bands. This suggested that the amount of antigen present in the control lung tissue was less than in the CFA tissue.

Figure 3.5

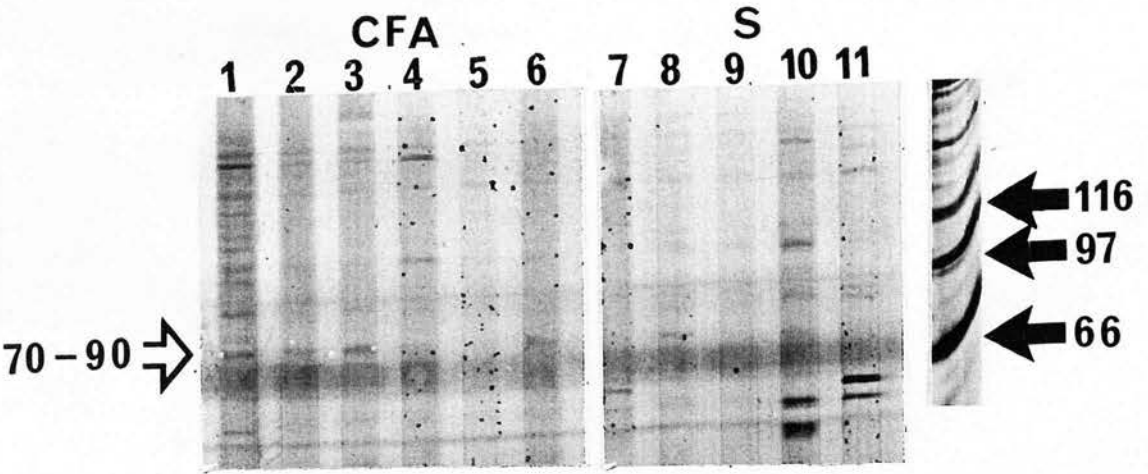


Figure 3.6

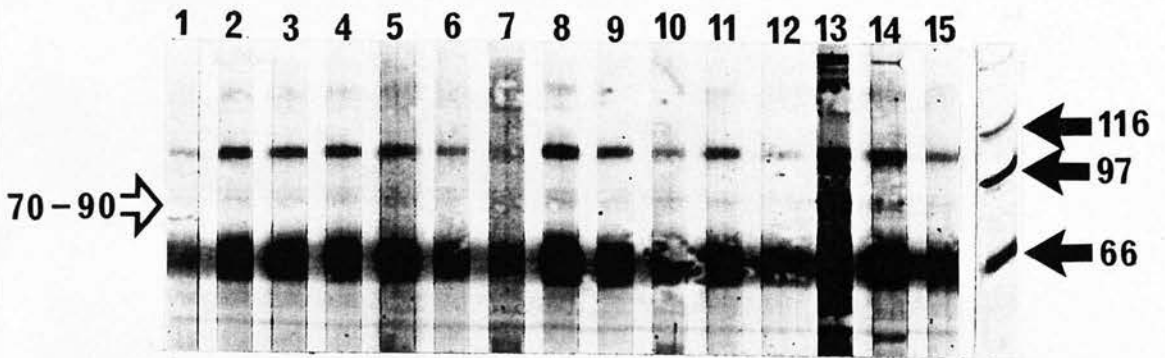


Figure 3.7

Illustrative data showing the results of blotting CFA plasma samples on protein extracts produced from (a) colonic mucosa and (b) liver using a monoclonal anti-human IgG γ as described in section 2.5. The molecular weight markers are shown on the right. Positive bands were detected in the 70-90 kDa region (arrow) in 3/13 (lanes 4,7 and 9) of the samples on the colonic mucosa and 3/14 (lanes 4, 9 and 10) on the liver.

Figure 3.7

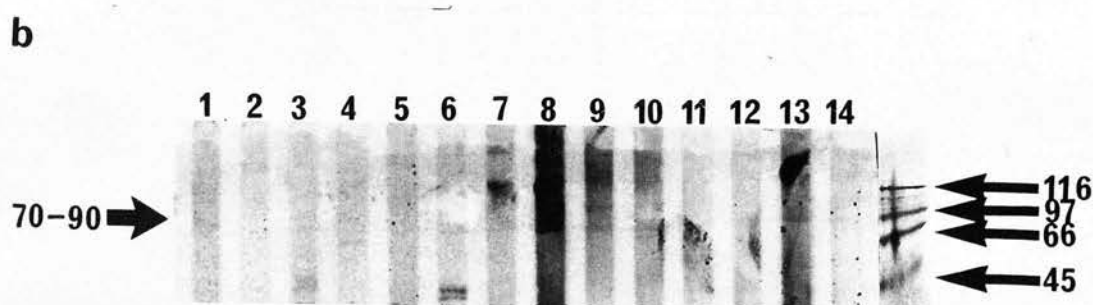
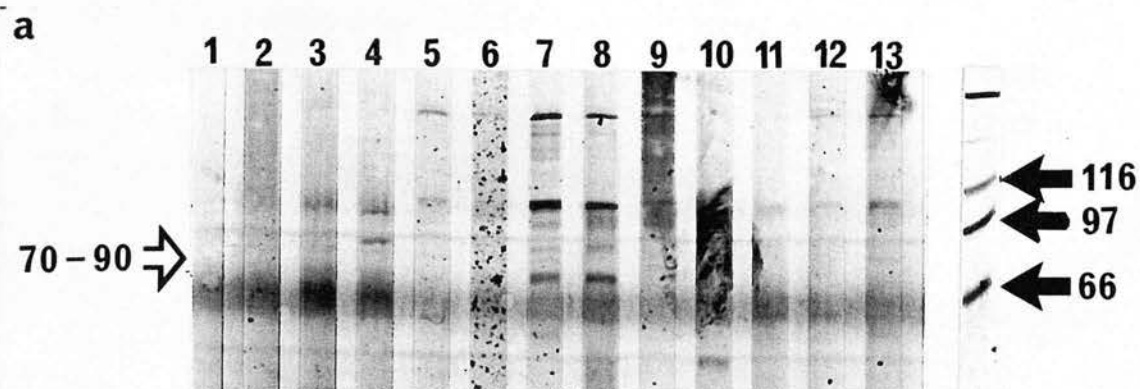


Figure 3.8

Results of a sandwich ELISA performed to compare the relative IgG content of the CFA, sarcoid and control plasma samples used in the Western blotting experiments. The ELISA was performed as described in section 2.10.1 and confirmed presence of elevated IgG levels in both patient groups compared with the controls although considerable overlap was evident. The patients with sarcoid, however, had the highest levels of IgG but despite this failed to have a higher frequency of antibodies to the 70-90 kDa lung protein than the healthy controls. This suggested that the results obtained with the CFA plasma samples were unlikely to simply be due to hypergammaglobulinaemia. (Mann-Whitney Test)

FIGURE 3.8

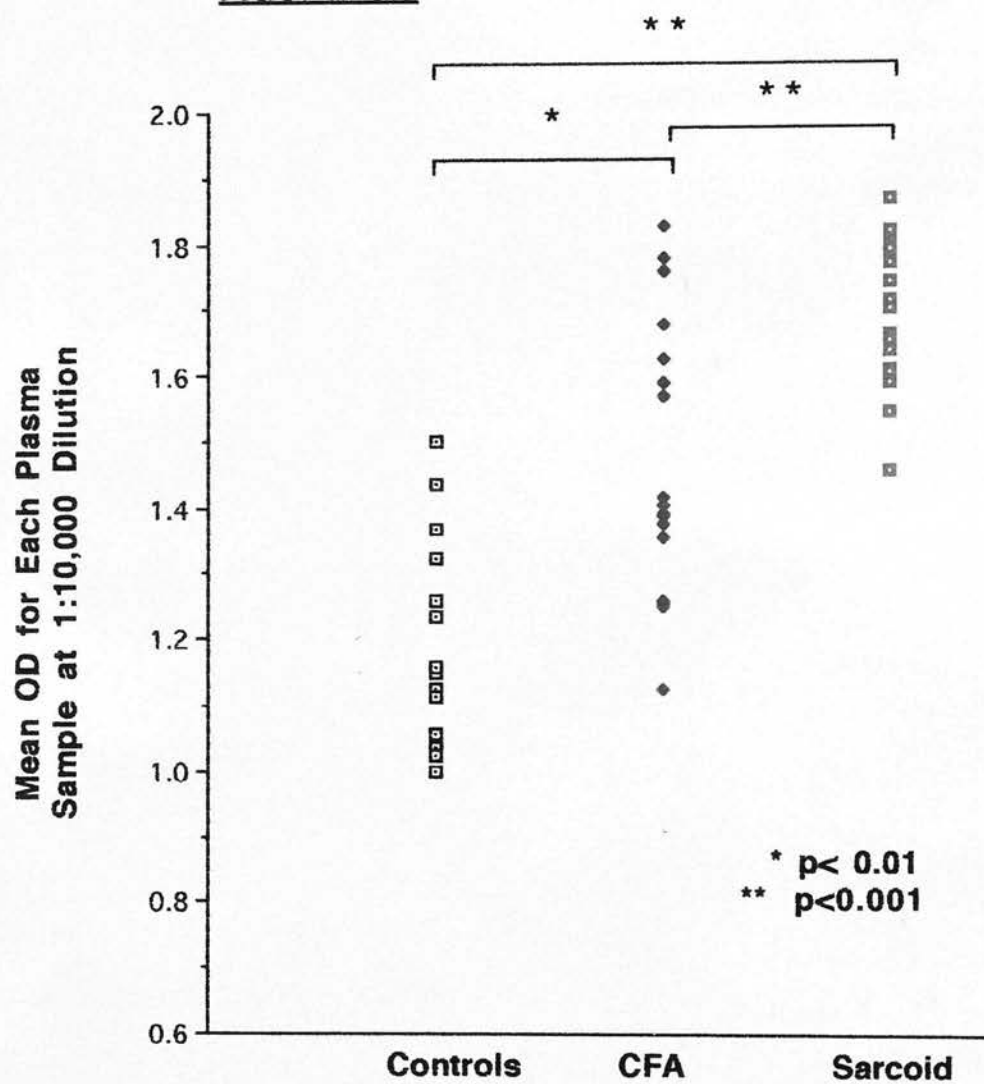
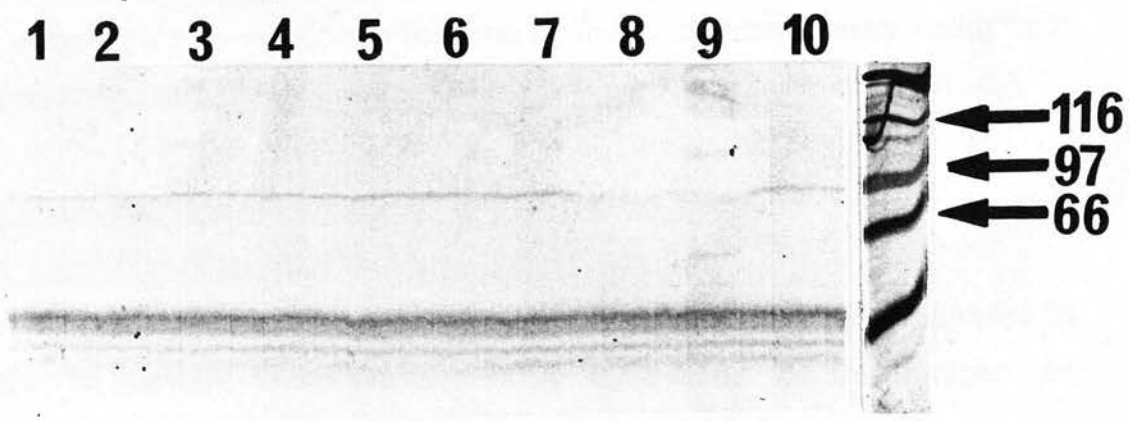


Figure 3.9

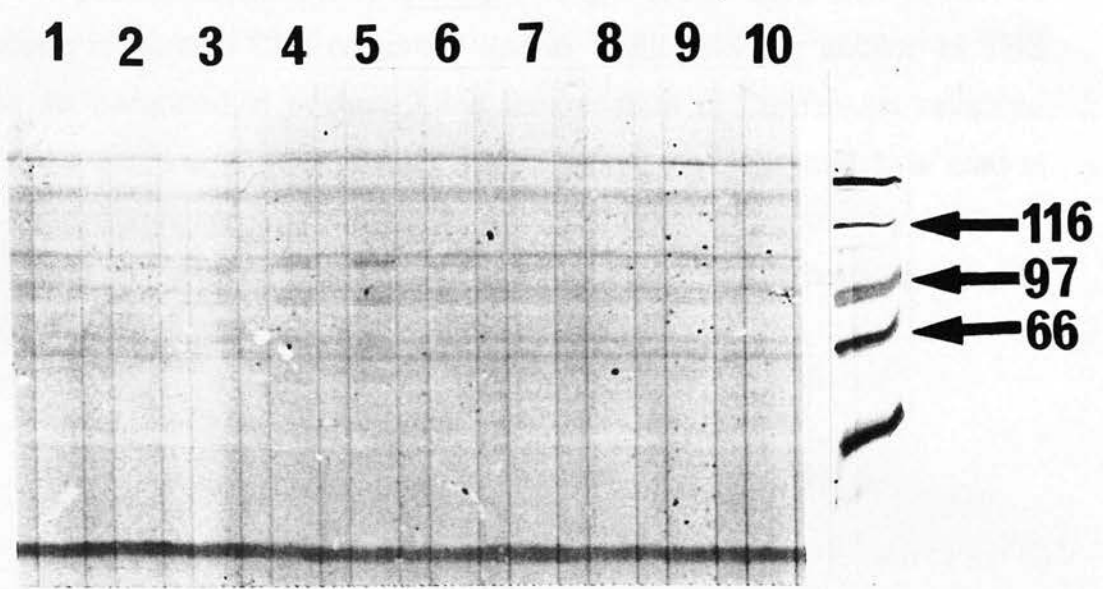
Representative Western blots performed to detect the presence of (a) IgM and (b) IgA in CFA plasma samples to the 70-90 kDa antigen previously detected in CFA lung tissue. The experiments were performed using material from a CFA open lung biopsy which had previously demonstrated to contain the antigenic protein. Detection was performed using a monoclonal anti-human IgM μ or anti-human IgA α as required using the methods described in section 2.5. While occasional positive bands were observed with both detection systems no evidence of IgM or IgA antibodies directed against protein(s) in the 70-90 kDa molecular weight range were detected.

Figure 3.9

IgM Detection



IgA Detection



Chapter 4

Detection of Pulmonary Antigenic Site with Purified Patient IgG.

The results of the Western blotting experiments in Chapter 3 indicated the presence of a 70-90 kDa autoantigen in lung tissue but gave no indication of its location. Experiments were therefore conducted in order to demonstrate its location in the lung by immunohistochemistry using both cryostat sections of CFA lung tissue and a type II alveolar cell line (A549).

4.1 Experiments with Purified IgG and CFA Lung Tissue.

4.1.1 Isolation of IgG and Western Blotting.

IgG was purified from 5 CFA patient and 4 control plasma samples in order to perform immunohistochemical localisation of the antigen as described in section 2.2.4.1. The concentration of IgG obtained was then estimated as described in section 2.2.3.1 and standardised. In order to confirm that the purified IgG contained the autoantibody Western blotting was performed. The results (Fig. 4.1) revealed that the IgG purified from the CFA but not the control IgG continued to recognise a 70-90 kDa antigen in CFA lung protein extracts.

4.1.2 Immunofluorescence.

Cryostat sections from 3 CFA open lung biopsies were stained with 50 or 100µg of purified CFA or control IgG in 100µl TBS per section or TBS alone as described in section 2.8.2. Examination of the results revealed extensive staining of all the sections including the TBS negative control indicating non-specific interaction of the FITC labelled secondary antibody with tissue elements on the slide. No specific staining could be detected with the CFA IgG that was not present in the controls.

4.1.3 Biotinylation of IgG.

In order to circumvent the problem of non-specific interactions of secondary reagents with tissue elements the purified IgG was directly biotinylated as described in section 2.2.4.2 so that detection could be performed without a secondary antibody. Biotinylation was performed on IgG samples obtained from 3 CFA patients and 2 controls. Dot blots were then developed with a horseradish peroxidase conjugated avidin biotin complex (HRP-ABC, Dako UK) and developed with diaminobenzidine (DAB) to demonstrate that the technique was successful.

Cryostat sections from 3 cases of CFA were then stained with a standard 50µg of the biotinylated IgG from either the CFA or control plasma sample or TBS. Adherent antibody was detected with ABC-AP complex and Vector Red as described in section 2.8.4.1. Examination of the sections by light and confocal laser microscopy revealed no positive staining of any of the sections including the negative control. Subsequent Western blotting with the biotinylated IgG revealed loss of reactivity to the 70-90 kDa antigen suggesting that the biotin may have blocked the antigen recognition sites on the antibodies.

4.1.4 Visualisation with Alkaline Phosphatase Conjugated Protein A.

Protein A is a bacterial derived protein which specifically binds immunoglobulin by the Fc portion of the molecule. By using such a reagent it was hoped this would eliminate non-specific interaction with cell bound immunoglobulin in the lung as these molecules would have their Fc receptors hidden. In order to block reaction with free immunoglobulin the sections were first treated with unconjugated Protein A, to block Fc receptors, followed by fresh normal human serum to block any interaction between the test IgG and protein A on the slide. The sections were stained with either TBS, control or CFA IgG as described in section 2.8.4.2.

Examination by light microscopy revealed no positive staining of the negative or control IgG sections. Very faint positive staining of alveolar

epithelial lining cells was, however, observed on the sections stained with the CFA IgG. This was confirmed when the sections were viewed by Confocal Laser Microscopy where prominent staining of the alveolar surface was observed to be present in the sections stained with CFA but not the control IgG (Fig. 4.2). The pattern of staining suggested that the antigen was associated with alveolar epithelial cells.

4.2 Comment.

Since it had been demonstrated that the autoantibody was an IgG this immunoglobulin subclass was purified from the plasma samples to try and decrease the potential for non-specific interactions during immunohistochemical experiments. Using purified IgG, rather than whole plasma, also had the advantage of allowing a standardised concentration of both the control and patient IgG for immunohistochemistry to control for any possible effect of the hypergammaglobuliaemia in the patient samples. Reactivity of the purified CFA IgG for the lung antigen was then confirmed by Western blotting to ensure that the relevant immunoglobulin fraction had been successfully purified.

The failure to identify specific staining using the immunofluorescence technique was not surprising. As has been discussed before this approach has previously been found to lack specificity because of non-specific interaction between the FITC conjugated anti-human antibody and immunoglobulin in the section.

The approach of avoiding the use of an anti-human immunoglobulin to detect adherent purified IgG seemed to provide a possible solution to this problem. Although the biotinylated IgG lost reactivity on the Western blots the Protein A detection system worked well with no apparent background positivity detectable on the negative control. Even with this technical problem overcome assessment by light microscopy remained difficult due to problems of image definition when examining weakly stained cells of only 2-3 μ m in diameter on a section which was 6-8 μ m in thickness.

In order to improve definition of the staining, sections were viewed on a confocal laser microscope (LSM). This instrument produces an optical section within the tissue being examined thus enhancing definition. In addition Vector Red is recognised to be more sensitive when viewed under uv compared with normal light. Thus using the LSM in uv mode resulted in removal of many of the optical limitations presented by standard light microscopy.

The demonstration that the CFA IgG autoantibody appeared to recognise an antigen associated with the alveolar epithelial cells was of particular interest because as discussed above this is believed to be the site of immunological injury in CFA (Corrin et al 1985). Further definition of the distribution of the antigen that the autoantibody was recognising was, however, not possible because of the poor morphological quality of the cryostat sections.

In order to further investigate the possibility that the 70-90 kDa antigen detected by the IgG autoantibody in the CFA patients sera was associated with alveolar epithelial lining cells, a type II alveolar epithelial cell line (A549) was obtained (Lieber et al 1976).

4.3 Experiments with Purified IgG and A549 Cells.

4.3.1 Western Blotting.

SDS-PAGE was performed using protein extracts obtained from the A549 cells. Western blotting was then performed as described in section 2.5 using plasma samples from 17 patients with histologically confirmed CFA [11M:6F, median age (range): 68(49-80)] and 17 healthy volunteers within the Department of Pathology who had no evidence of respiratory symptoms and were on no medication [10M:7F, median age (range): 42(28-58)].

12/17 (71%) of the patients had IgG which recognised a protein in the 70-90 kDa range on the blot compared with only 3/17 (18%) (Fig. 4.3) of the healthy control plasma samples ($p < 0.01$, Chi Square Test). This frequency of positivity in the 70-90 kDa molecular weight range was not

significantly different from that observed with CFA lung protein extracts for either group. In comparison when CFA plasma samples were blotted on extracts derived from the Jurkat and H9 lymphoid cell lines a significantly lower frequency ($p < 0.01$, Chi Square Test) was observed (Jurkat 1/10, H9 2/10).

4.3.2 Immunohistochemistry.

Cytospin preparations of A549 cells prepared as described in section 2.3.1.1 were stained with IgG purified from CFA patients and controls using the alkaline phosphatase conjugated Protein A method as described in section 2.8.4.2. This revealed positive cytoplasmic staining with the patient IgG but not the control IgG (Fig. 4.4).

4.3.3 Immunoabsorption.

In order to confirm that the antigen present in the A549 cells detected by the patient autoantibody was the same as in the lung tissue extracts immunoabsorption experiments were performed. A pooled sample of IgG obtained from 4 patients with CFA was absorbed against harvested A549 cells which had been disrupted by repeated rapid freezing and thawing. Following absorption at 4°C overnight the cell debris was removed by centrifugation, the IgG recovered with Protein G (section 2.4.1) and the concentration estimated and standardised.

Western blotting with absorbed and unabsorbed CFA patient IgG was then performed with protein from a CFA open lung biopsy in order to determine if reactivity against the 70-90 kDa antigen had been removed. The results (Fig. 4.5) showed a reduction in the intensity of staining following absorption suggesting that some of the IgG recognising antigen of this weight had been removed by absorption with the A549 cells.

4.4 Comment.

The A549 cell line was originally derived from a bronchoalveolar carcinoma and as such is fully transformed. These tumours are believed to arise from type II alveolar lining cells and the cell line has been demonstrated to show features characteristic of these parent cells (Lieber et al 1976). The Western blotting and immunohistochemical results obtained thus provided further evidence that the antigen of interest was expressed by alveolar epithelial cells.

In order to confirm that the 70-90 kDa antigen expressed by the cell line was identical to that detected in the lung extracts immuno-absorption experiments were conducted. The CFA plasma samples were absorbed against freeze/thaw fractured A549 cells rather than whole cells as the immunohistochemical data had suggested that the antigen had a predominantly cytoplasmic distribution. Although absorption did not remove reactivity against the lung derived 70-90 kDa antigen completely it was substantially attenuated suggesting that at least some of the antigen recognised by the patient plasma was present in the A549 cell line. Further experiments confirming that the antigen detectable in the A549 cells is the same as that found in the lung tissue are presented in Chapter 6. These results strongly supported the observations made on the tissue sections which indicated that the auto-antigen had an alveolar epithelial distribution.

4.5 Conclusion.

The antibody detected in plasma samples from patients with CFA was demonstrated to recognise an antigen associated with alveolar epithelial lining cells in lung biopsy specimens. The antibody also appeared to recognise the same antigen expressed in the A549 type II epithelial cell line.

Figure 4.1

Western blot illustrating the results of blotting purified IgG from CFA and control plasma samples against protein derived from CFA lung biopsy tissue developed using an anti-human IgG γ as described in section 2.5. Bands of varying intensity can be identified in the 70-90 kDa molecular weight range (arrow) in the lanes corresponding to the CFA patient derived IgG but not in lanes with control IgG at the same concentration.

Figure 4.1

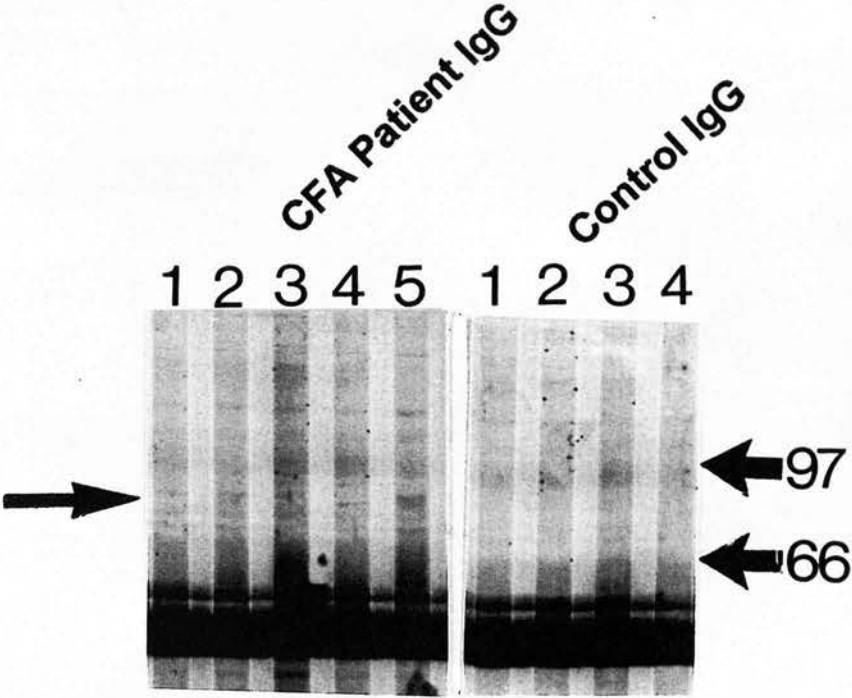
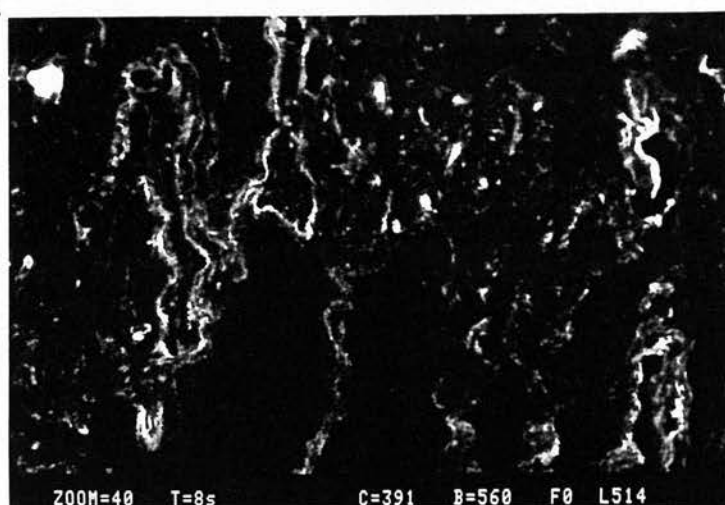


Figure 4.2

Photomicrograph obtained using the Confocal Laser Microscope in uv mode of a cryostat section from a CFA lung biopsy stained with purified IgG from (a) control plasma and (b) CFA patient plasma. Staining was performed using the protein A conjugated immuno-alkaline phosphatase method described in section 2.8.4.2. In the section stained with the control IgG background auto-fluorescence from the collagen and elastin structures in the lung parenchyma can be seen. In contrast sections from the same case stained with the CFA patient IgG show linear positivity along the edge of the alveoli. This pattern of staining suggests that the antigen(s) recognised by the patient IgG are associated with alveolar lining cells. (Original magnification (a) X40, (b) X100)

Figure 4.2

a



b

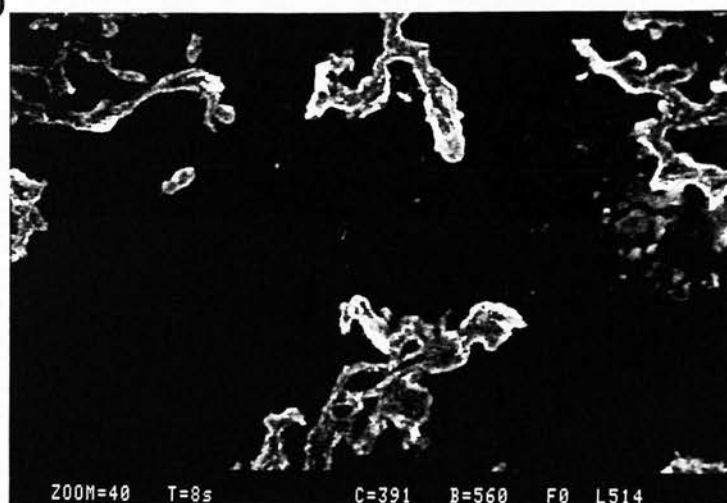
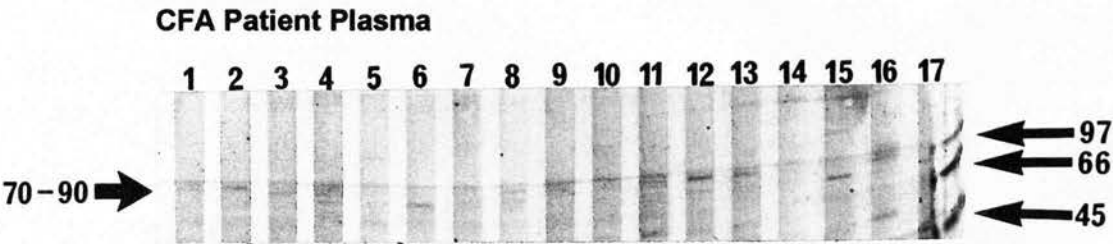


Figure 4.3

Representative Western blots illustrating the results obtained blotting (a) CFA patient plasma and (b) non-smoking healthy control plasma samples on protein extracts derived from A549 cells. The blots were developed using an anti-human IgG γ as described in section 2.5. The molecular weight markers are indicated in kDa on the right of both blots. Positive bands are identified in the 70-90 kDa range (arrow) in 12/17 of the CFA plasma samples (lanes 1, 2, 3, 4, 6, 8, 11, 12, 13, 15, 16 and 17) compared with only 3/17 with the control plasma (lanes 13,15,16). ($p < 0.01$, Chi Square Test)

Figure 4.3

a



b

Control Plasma

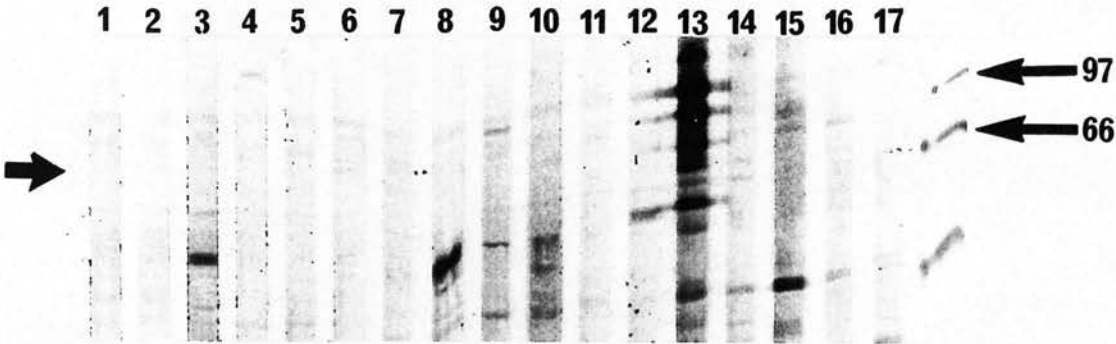
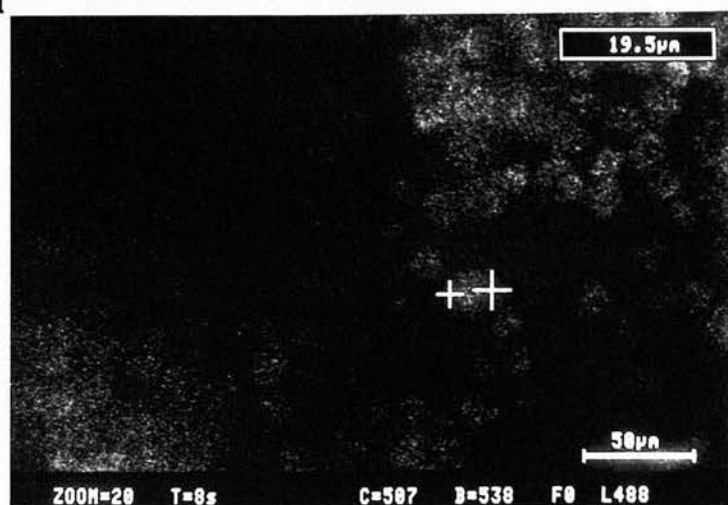


Figure 4.4

Photomicrograph obtained using the Confocal Laser Microscope in uv mode of cytopsin preparation of A549 cells stained with purified IgG from (a) control plasma and (b) CFA patient plasma. Staining was performed using protein A conjugated immuno-alkaline phosphatase method as described in section 2.8.4.2. Cells stained with the control IgG showed no specific positivity, however those stained with the CFA patient IgG showed evidence of cytoplasmic staining. (Original magnification (a) and (b) X200)

Figure 4.4

a



b

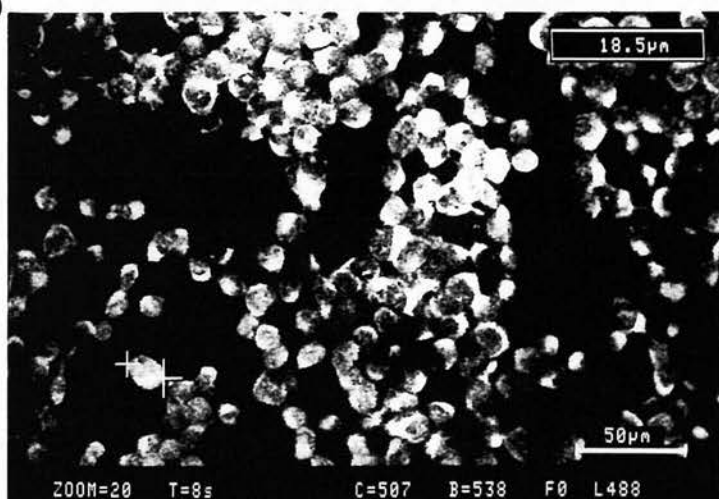
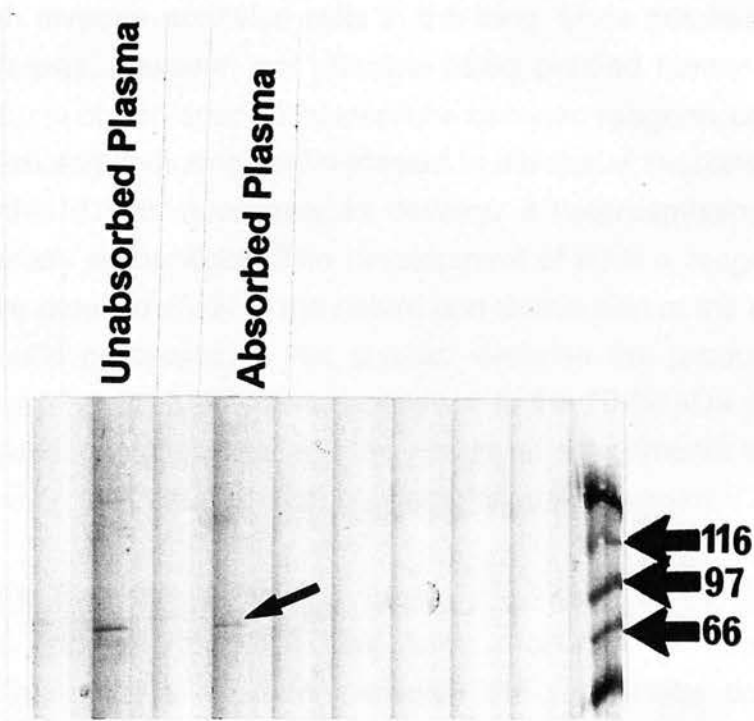


Figure 4.5

Western blot illustrating the results obtained blotting a pooled sample of CFA patient IgG on protein derived from CFA lung tissue with and without prior absorption with freeze/thaw fractured A549 cells as described in section 4.2.3. The blots were developed using an anti-human IgG γ as described in section 2.5. The molecular weight markers are indicated in kDa on the right. The position of the reactive bands in the 70-90 kDa molecular weight range is indicated (arrow). The intensity of the band obtained with the absorbed plasma is less than with the original despite applying a standard concentration of IgG to each lane. This suggests that, at least in part, antibody recognising the 70-90 kDa CFA lung antigen has been removed by prior absorption with the A549 cells and therefore that the antigens may be the same.

Figure 4.5



CHAPTER 5.

Characterisation of a Heteroantiserum and its use to Study the Tissue and Cellular Distribution of a 70-90 kDa Pulmonary Autoantigen.

The results obtained in Chapter 4 indicated that the antigen was associated with alveolar epithelial cells in the lung. More detailed study of the distribution was, however, not possible using purified human antibody due to the problem of non-specific interactions between reagents used in the detection system and immunoglobulin present in the tissue sections. In order to overcome this it was necessary to develop a heteroantiserum to the putative pulmonary autoantigen. The development of such a reagent would then allow more detailed study of the nature and distribution of the antigen.

The results presented in this chapter describe the production and characterisation of a rabbit polyclonal antiserum to the 70-90 kDa pulmonary antigen previously demonstrated and the results of experiments conducted using it to examine the tissue and cellular location of the antigen.

5.1 Production of Rabbit Anti-Serum.

Immune and control rabbit sera were produced as described in section 2.6. The material used to immunise the rabbit was deliberately obtained by two methods as the antigenicity of the material and yield obtained by either method alone was unclear.

5.1.1 Immunoaffinity Purification of Lung Antigen

Immunoaffinity purification of the lung antigen was performed as described in section 2.6.1 using IgG obtained from patient plasma samples coupled to cyanogen bromide activated sepharose beads. The eluent obtained from the column had a very low protein content, below the sensitivity of the protein assay (0.05mg/ml) and despite subsequent experiments using larger volumes of beads and IgG, could not be increased. Immunoblotting, following absorption of the eluent with Protein G to remove human IgG stripped from the column, confirmed that an antigen was obtained that was not IgG and which was recognised by CFA but not control plasma samples. The yield of antigen obtained with this method was insufficient to allow SDS-PAGE to be performed but nevertheless provided complexes of the putative auto-antigen for immunisation of the rabbit.

5.1.2 Partial Purification of Lung Antigen by SDS-PAGE.

The putative autoantigen was also partially purified from CFA lung biopsy tissue using SDS-PAGE as described in section 2.6.2. This method was utilised to provide antigen of the correct molecular weight for immunisation.

5.1.3 Immunisation.

The rabbit was immunised and test bled as described in section 2.6.3. The IgG from each sample was then purified, absorbed and standardised at a concentration of 1mg/ml as described in section 2.6.4.

5.2 Characterisation of Immune Rabbit IgG.

5.2.1 Western Blotting.

The immune and control rabbit sera were blotted against protein derived from CFA lung biopsies and A549 cells using the methods described in sections 2.4 and 2.5. The immune IgG was shown to recognise a protein in both extracts in the 70-90kDa region neither of which was detected by the control IgG (Fig. 5.1). The reactivity to the protein showed a dose dependent response with decreasing intensity of staining observed when less IgG was applied.

5.2.2 Immunoprecipitation.

Immunoprecipitation was performed using protein extracts pooled from 4 open lung biopsies from patients with CFA as described in section 2.7. The resultant material obtained was run on SDS gels as described in section 2.4 with the original protein mixture along side for comparison. Coomassie blue staining (Fig. 5.2) revealed intense protein bands in the 55 and 110 kDa weight range which corresponded to the expected molecular weight of the rabbit IgG heavy chain existing in monomeric and dimeric forms. Several faint bands of lower molecular weight were also observed which corresponded to the expected position of light chains. In addition distinct bands at c.80 and 160 kDa were also identified. These were in the positions predicted for the pulmonary antigen existing either in monomeric or dimeric forms from the Western blotting experiment performed with the immune IgG (Figure 5.1).

Western blotting (Fig. 5.3) confirmed that the 80 and 160 kDa proteins showed cross reactivity with CFA patient plasma. The monoclonal

anti-human IgG γ antibody used in this detection was, however, known to cross react with rabbit IgG and so a parallel blot with the human plasma omitted was performed. This showed no reactivity at these positions in the absence of the CFA plasma but did detect the 55 and 110 kDa proteins. This confirmed that the 55 and 110 kDa bands were IgG while the 80 and 160 kDa were not and thus must have been precipitated from the lung protein extract.

These experiments confirmed that the immune rabbit IgG recognised a pulmonary antigen of the same molecular weight as the putative auto-antigen and that the protein precipitated showed cross reactivity with the patient autoantibody.

5.2.3 Immunoabsorption.

Immune rabbit sera was absorbed with freeze/thaw fractured A549 cells and the IgG purified in the same manner as had been used for the CFA patient plasma (Chapter 4). Western blotting performed with the absorbed IgG revealed complete loss of reactivity to the 70-90 kDa antigen on extracts of CFA lung biopsy tissue (Fig. 5.4). Parallel experiment conducted absorbing the rabbit serum with fractured Jurkat cells, which do not express the 70-90 kDa antigen (see section 4.3.1) revealed no such loss of reactivity (Fig. 5.5). These experiments confirmed that the immune rabbit serum contained IgG recognising the same protein in both the A549 cells and lung extracts.

5.2.4 Antigen Capture Experiments.

In order to determine whether the patient autoantibody and the rabbit IgG were directed against the same protein an antigen capture experiment was devised. Since lung tissue contains large quantities of immunoglobulin which would have interfered with the detection system such experiments were performed only with protein derived from the A549 cell line.

Antigen capture experiments were conducted as described in section 2.10.2. Immune rabbit serum was coated onto the ELISA plate and following blocking was incubated with 50 μ g of A549 cell protein. The plates were then incubated with either plasma from 4 patients with CFA or 2 controls in triplicate at doubling dilutions. The plates were developed for adherent human IgG and read. The results (Fig. 5.6) indicated a concentration dependent binding of human IgG in the CFA samples which was absent in

the controls. This suggested that both the human CFA IgG and the immune rabbit IgG both contained antibodies which recognised the same antigen present in the A549 protein extracts.

5.3 Comment.

Development of the anti-human sera using only partially purified antigen obtained from CFA lung material was considered the most likely method of producing a useful reagent to further study the putative human auto-antibody. By purifying and absorbing the control and immune IgG as described artefacts related to either anti-human IgG activity or variations in IgG concentration were eliminated.

The Western blotting experiments with the CFA lung extract indicated that an immune response to a pulmonary antigen of the appropriate molecular weight had been achieved. This was confirmed by immunoprecipitation. The observation that the rabbit IgG recognised a protein of the same molecular weight in the A549 cell extracts and that the precipitated lung antigen showed cross reactivity with CFA plasma strongly suggested that the animal had been successfully immunised against the putative auto-antigen.

The immuno-absorption experiments indicated that the rabbit sera appeared to recognise the same A549 antigen as the human CFA autoantibody. When this result was combined with the observation made in Figure 4.5 and 5.4 indicating that both the human and rabbit antibodies to the 70-90 kDa antigen recognise the same antigen in the A549 cells and CFA lung biopsy extracts, it was concluded that the rabbit serum contained an antibody recognising the same pulmonary antigen as the patient autoantibody. The immuno-absorption results with the rabbit sera also confirmed the fact that the A549 antigen was the same as that detectable in the lung tissue extracts. This then allowed a series of immunohistochemical experiments to be conducted in order to further localise the antigen.

5.4 Immunohistochemical Localisation of Antigen.

Experiments with formalin fixed, paraffin embedded sections from the departmental archives revealed no reactivity despite pre-treating the sections with trypsin or microwave heating as described in sections 2.8.1.1 and 2.8.1.2. All immunohistochemical staining of tissue sections was therefore carried out on frozen material using an ABC-alkaline phosphatase

method visualised with Vector Red as described in section 2.8.4.1. Sections from all cases were stained with control IgG or immune IgG at a 1:10 (100µg/ml) dilution in TBS or TBS alone.

5.4.1 CFA Lung biopsy Tissue.

Cryostat sections from 8 cases of CFA were stained as described and all showed a similar pattern of reactivity. No positivity was observed in the sections stained with control IgG (Fig.5.7) or buffer alone. The results with the immune IgG (Fig. 5.8) revealed prominent staining of the alveolar epithelial lining cells in the tissue. The pattern was similar to that which had been previously observed with the human IgG (Chapter 4) being linear along the alveolar surfaces in a pattern suggesting the antigen was associated with type I alveolar epithelial lining cells. In areas where there were hyperplastic type II epithelial cells the staining was cytoplasmic. Bronchial epithelial cells showed no specific cellular staining but patch positivity of the cilia (Fig. 5.9) was observed suggesting that antigen might be present in the airspaces and trapped in the muco-ciliary transport system. Occasional macrophages were also noted to show cytoplasmic positivity (Fig. 5.10).

Assessment of the distribution of positive staining in relation to disease activity proved difficult on the cryostat sections. Staining appeared most prominent in areas of fibrotic lung with obvious type II cell hyperplasia but this was not uniform even within cases. Staining was also evident in areas of CFA biopsies with apparent normal alveolar septa at the edge and focally in areas of dense fibrosis. No clear correlation could be identified between positive staining with the immune IgG and disease activity.

5.4.2 Sarcoid Lung Biopsy Tissue.

Sections from 2 open lung biopsies from patients with sarcoidosis were similarly stained (Fig. 5.11). The immune IgG again stained alveolar epithelial lining cells. This was most prominent where hyperplastic epithelium could be observed adjacent to granulomata. In other areas the staining was less intense and more focal. Occasional alveolar macrophages were again noted to be positive. Staining of giant cells and macrophages within granulomata was observed with the immune IgG although the significance of this was unclear as faint positive staining of these cells was also apparent on section stained with the control IgG but not buffer alone.

5.4.3. Control Lung Tissue.

Tissue from 2 pneumonectomy specimens performed for malignant disease was obtained to provide control pulmonary tissue. The sections did not contain either tumour or complications of the tumour and nor were histological features suggesting any form of ILD seen. Immunohistochemistry with the immune rabbit IgG revealed focal linear staining of the alveolar surface which was less uniform and intense than that observed on the CFA sections (Fig. 5.12). The pattern was again suggestive of reactivity with an antigen associated with type I alveolar epithelial cells. Due to the poor morphology of the cryostat sections type II cells could not be readily identified in these sections.

5.4.4 A549 Cell Line.

Cytospin preparations of A549 cells produced as described in section 2.3.1.1 were similarly stained. The immune IgG showed cytoplasmic staining of the cells (Fig. 5.13) in a similar manner to the patient IgG (Chapter 4).

5.4.5 Non-Pulmonary Tissues.

Cryostat sections were obtained from histologically normal skin, tonsil, lymph node, colonic mucosa, cervix, liver and kidney (representative data Fig. 5.14). The sections were stained as described in section 2.8.4.1 with the inclusion of A549 cytopins in each experiment to act as a positive control. No positive staining was observed on any of the tissues with either the control or immune IgG although appropriate staining was observed with the A549 cells.

5.4.6 Peripheral Blood Mononuclear cells and Other Cell Lines.

Cytospin preparations of peripheral blood mononuclear cells, human T cell lines (H9 and Jurkat) and primary cultured chondrocytes were obtained and stained as described in section 2.8.4.1. No positivity was observed with the immune IgG with any of the cells although some patchy non-specific staining of the matrix surrounding the cultured chondrocytes was observed with both the control and immune IgG.

5.5 Comment.

The results obtained with the immune rabbit IgG appeared consistent with the observations made with the patient IgG in Chapter 4 and confirmed that the distribution of the 70-90 kDa auto-antigen in the lung was related to the alveolar epithelial lining cells. This result appeared to be reproducible in 8 cases of CFA and was associated with no similar staining in the negative controls thus excluding any possible confounding effect of endogenous alkaline phosphatase activity.

The results were also entirely consistent with the Western blotting data presented in Chapter 3 in that the antigen was detectable in pulmonary tissue from patients with sarcoidosis and normal control lung tissue. The intensity of the staining in the non-ILD tissue was however considerably less than in the ILD lung tissue supporting the observation that the antigen may be upregulated in the CFA and sarcoid tissue. These observations again supported the conclusion drawn in Chapter 2 that the antigen may be endogenous.

The distribution of epithelial staining was not uniform within biopsy specimens and appeared most obvious where type II cell hyperplasia was evident. It was unclear whether this was simply that type II cells tended to stain more prominently because of their morphology or if this represents a true increase in positivity in such diseased areas. The staining of apparently normal alveolar septa in the CFA biopsies appeared more marked than in the non-ILD control lung tissue suggesting that the antigen may be up regulated in epithelia adjacent to the diseased areas as well as in damaged lung. The distribution of the antigen recognised by the immune rabbit IgG was difficult to correlate with local disease activity in the cryostat sections but the association with areas of type II cell hyperplasia suggests a possible link with activity. The inability of the antibody to work on paraffin sections, where studies of this kind would be more feasible, precludes further comment on this aspect of the distribution.

The antigen also appeared to have an epithelial distribution in the sarcoid lung biopsies examined. This appeared most prominent in the hyperplastic epithelium adjacent to granulomas. The giant cells and macrophages within the granulomas also appeared positive although some staining of these cells was also evident with the control IgG. The significance, therefore, of the giant cell and granulomatous macrophage

staining was unclear and it may represent a degree of non-specific staining due to increased Fc receptor expression by these activated cells.

The absence of any positive staining on the non-pulmonary tissues and cells with the immune rabbit IgG suggests that the antigen detected by the immune IgG may be lung specific and appears consistent with the Western blotting data presented in Chapter 3. As the majority (>90%) of CFA cases are not associated with a systemic condition it would seem probable that any antigen having a role in the pathogenesis of the disease may be lung specific. The demonstration that the 70-90 kDa protein may show such specificity was thus of major interest.

The presence of positive cytoplasmic staining in some alveolar macrophages and positivity along the ciliated edge of bronchial epithelial cells in the biopsies from the CFA patients suggests that the antigen may be released from the epithelial cells. This could result from either the antigen being a secretory product or as a result of cellular injury in the disease process.

The development of the heterologous antisera and the demonstration that the antigen was expressed by the A549 cell line allowed further experiments using flow cytometry and differential extraction of microsomal and cytoplasmic proteins to be performed in order to study the cellular distribution of the antigen thus giving some clue as to its possible nature.

5.6 Flow Cytometry.

Flow cytometry was performed on A549 cells using control and immune rabbit IgG with and without permeabilisation of the cell membrane as described in section 2.9.1. A monoclonal antibody to vimentin, an intracellular cytoskeletal element, was used as a positive control. In the non-permeabilised cells stained with the immune IgG a sub-population showed some positivity. The reason for this is unclear. It could represent a low level surface expression by some cells or may indicate loss of viability and membrane integrity. The latter explanation seems less likely as all the data were collected by gating on a single viable cell bitmap and no alteration in the scatter profile was observed. The results with the permeabilised cells confirmed the immunohistochemical impression that the antigen was predominantly cytoplasmic in nature. Interestingly the profile obtained after permeabilisation with the immune IgG showed an apparent double peak

suggesting two populations of cells with differing levels of antigen expression. The significance of this is unclear.

5.7 Western Blotting with Microsomal and Cytoplasmic Protein Derived from A549 Cell Lines.

Differential extraction of microsomal and cytoplasmic proteins from A549 cells was performed as described in section 2.3.2.2 and the protein yield estimated. Equal amounts of microsomal, cytoplasmic and protein extracts derived from whole A549 cells with NP-40 as a control were run in parallel on 7.5% SDS gels under reducing conditions. Western blotting was then performed with both immune rabbit IgG and CFA plasma pooled from 4 patients.

The results indicated that the antigen detectable in the whole cell extract in both blots was predominantly associated with the microsomal compartment of the cell (Fig. 5.16). Some faint positivity was apparent at the same position in the cytoplasmic fraction but this may simply represent contamination in the preparation of the extracts.

5.8 Comment.

Immunohistochemical studies with both the human CFA autoantibody (Chapter 4) and the immune rabbit IgG had suggested that the antigen appears to have a predominantly cytoplasmic distribution in the A549 cells and this was confirmed by flow cytometry and by the observation that the antigen appeared associated with the microsomal fraction of the cell. The possibility that a low level of cell membrane expression is present on viable cells could be investigated by flow cytometry of unfixed cells stained both with the immune IgG and propidium iodide.

It is recognised that type II alveolar epithelial cells export proteins into the airspaces. Proteins that are produced by the cell for export, or for surface expression, are recognised to be post-translationally modified and stored in the microsomal organelles within cells. Thus if the antigen is a secretory product of the type II cells then the staining identified above in association with the type I cells may result from antigen in the alveolar lining fluid rather than actually in or on the cells themselves.

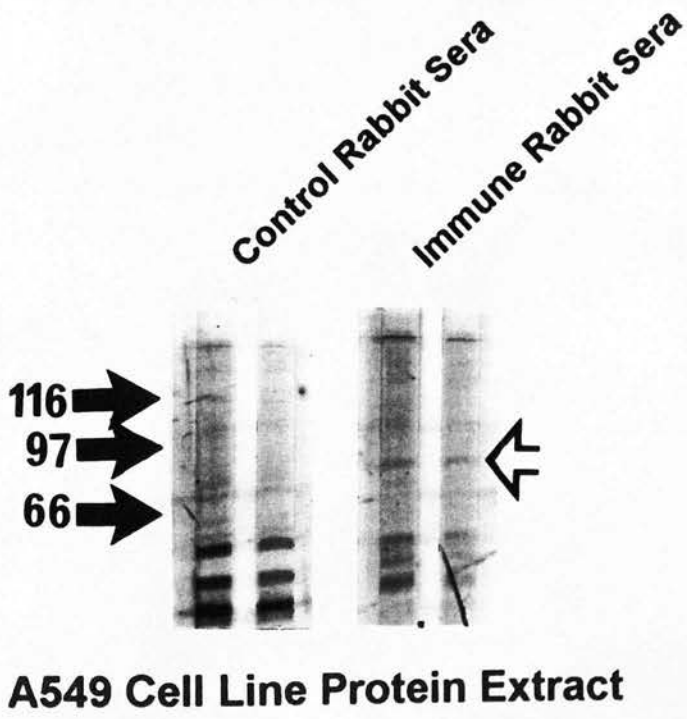
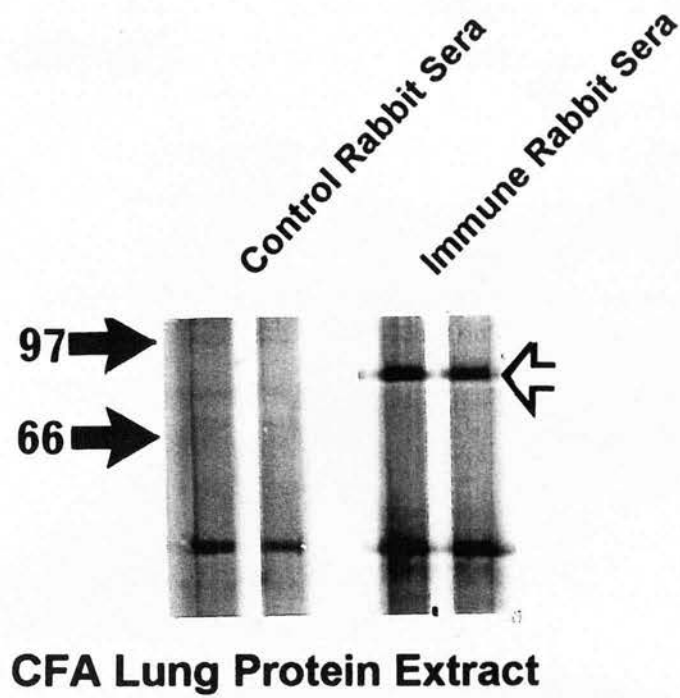
5.9 Conclusion.

The heteroantiserum developed appears to recognise the same pulmonary autoantigen as the patient IgG autoantibody. Immunohistochemistry suggests that this antigen is restricted to the lung and expressed in association with alveolar epithelial lining cells. In the A549 cell line the antigen appears to be predominantly intracellular, associated with the microsomal fraction of the cell.

Figure 5.1

Illustration of Western blotting results obtained using control rabbit (pre-immunisation) sera and immune sera on protein extracts obtained from C lung tissue and A549 cells. The blots were developed using a biotinylated swine anti-rabbit antibody as described in section 2.5. The molecular weights are indicated on the left. The immune sera, but not the control, recognised antigen in the 70-90 kDa molecular weight range in both protein extracts.

Figure 5.1



111a

Figure 5.2

Polyacrylamide gel stained with Coomassie blue illustrating the results obtained by immunoprecipitation of antigen from CFA lung protein extracts using the immune rabbit IgG as described in section 2.7. The left lane shows the position of bands obtained following disassociation of the precipitated human proteins from the immune rabbit IgG. The banding pattern obtained with the protein extract used in the experiment is shown alongside for comparison and the molecular weight markers are shown on the right. Bands of 55 and 110 kDa are observed which are believed to correspond to the rabbit IgG γ chains. In addition fainter bands at around 80 and 160 kDa are present which may represent monomeric and dimeric forms of the pulmonary protein which the rabbit antibody was raised to.

Figure 5.3

Illustration of the results obtained blotting protein immunoprecipitated from CFA lung biopsy protein extracts in the presence and absence of CFA patient plasma using a monoclonal anti-human IgG γ as described in section 2.5. Such a step was necessary as it was recognised that the anti-human IgG γ showed cross reactivity with rabbit IgG. The molecular weight markers are indicated on the right. The bands in the 50 and 110 kDa range showed positivity in both blots confirming their nature as rabbit immunoglobulin, probably IgG heavy chains in monomeric and dimeric forms. The bands in the 80 and 160 kDa range were, however, only detected in the presence of patient plasma indicating that they were not fragments of the rabbit IgG and were therefore precipitated lung proteins of the appropriate molecular weight for those recognised by the immune rabbit serum (Figure 5.1).

Figure 5.2

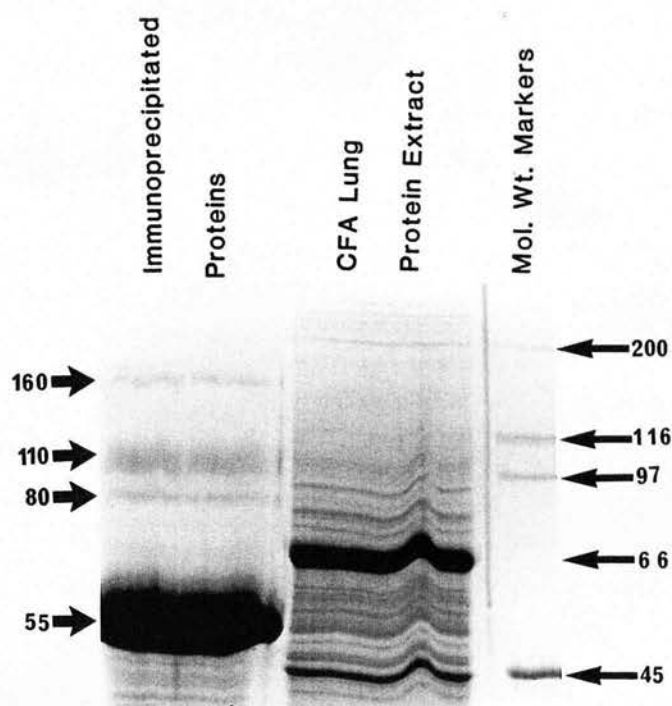


Figure 5.3

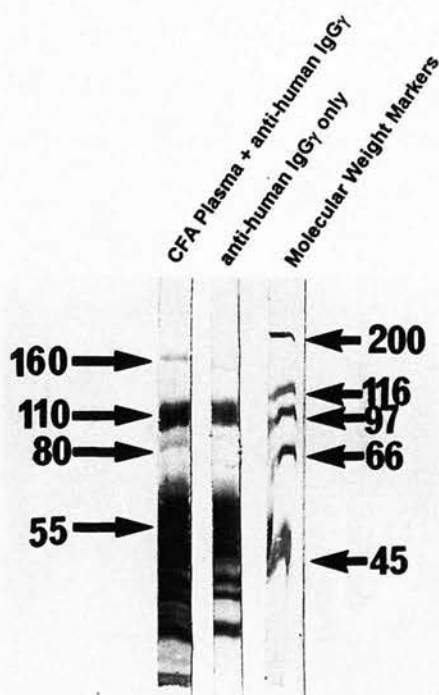


Figure 5.4

Illustration of Western blotting results obtained with immune rabbit IgG on protein derived from CFA lung tissue with and without prior absorption with freeze/thaw fractured A549 cells as described in section 4.2.3. The blots were developed using a biotinylated swine anti-rabbit immunoglobulin as described in section 2.5. The molecular weight markers are indicated in kDa on the right. The position of the reactive bands in the 70-90 kDa molecular weight range is indicated (arrow) with the unabsorbed IgG. No corresponding band is identified using the absorbed IgG at an identical concentration implying that the antibody present in the rabbit IgG had been removed by prior absorption with the A549 cells. This, therefore suggested that the 70-90 kDa antigen present in the lung extracts was the same as in the A549 cells. Similar results were also obtained in Chapter 4 using IgG purified from CFA patients.

Figure 5.5

Illustration of Western blotting results obtained with immune rabbit IgG on protein derived from CFA lung tissue with and without prior absorption with freeze/thaw fractured Jurkat cells as described in section 4.2.3. The blots were developed using a biotinylated swine anti-rabbit immunoglobulin as described in section 2.5. The molecular weight markers are indicated in kDa on the right. The position of the reactive bands in the 70-90 kDa molecular weight range is indicated (arrow) with the unabsorbed IgG. In contrast to the result obtained absorbing the immune rabbit IgG with A549 cells (Figure 5.4), no apparent loss of reactivity was observed using the Jurkat cells which are recognised not to express the 70-90 kDa antigen.

Figure 5.4

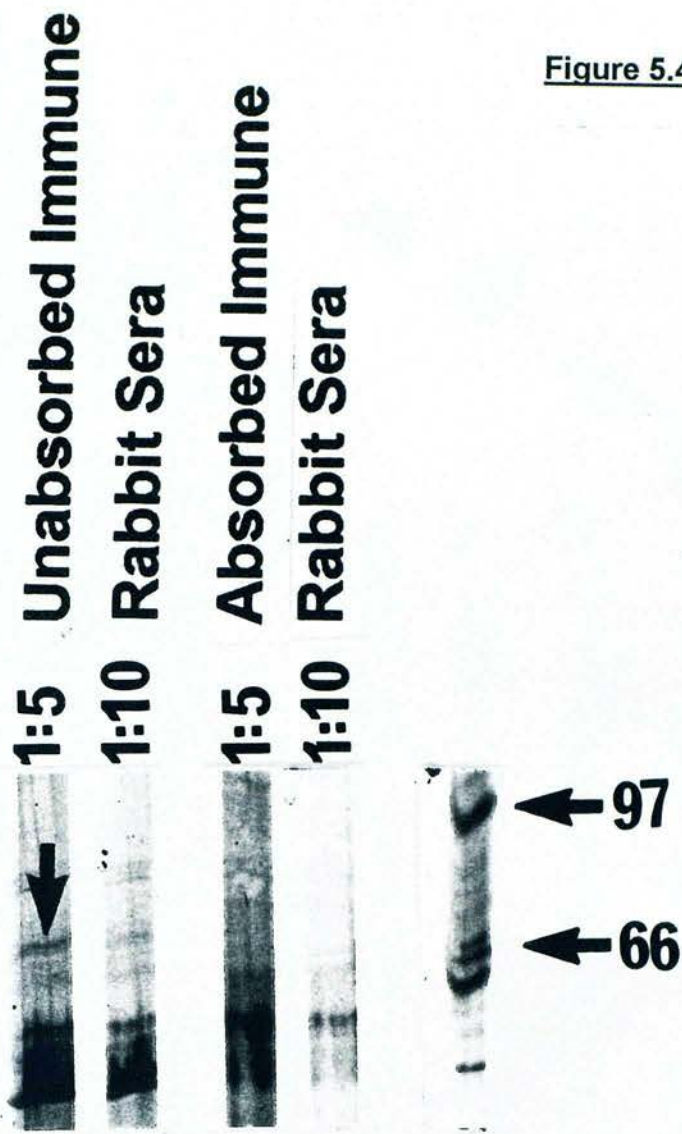


Figure 5.5

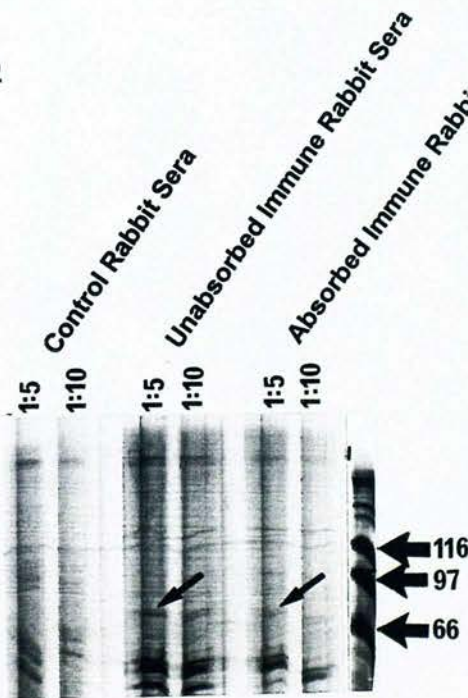


Figure 5.6

Illustration of results obtained during antigen capture experiments with immune rabbit IgG, A549 protein extracts and control or CFA patient plasma as described in section 2.10.2. The results showed evidence of concentration dependent binding of CFA patient IgG to the captured antigen on the plate but no similar pattern with the control samples. These results indicated that the human IgG autoantibody and the immune rabbit IgG were both recognising the same antigen which was present in the A549 cell protein extract.

Figure 5.6

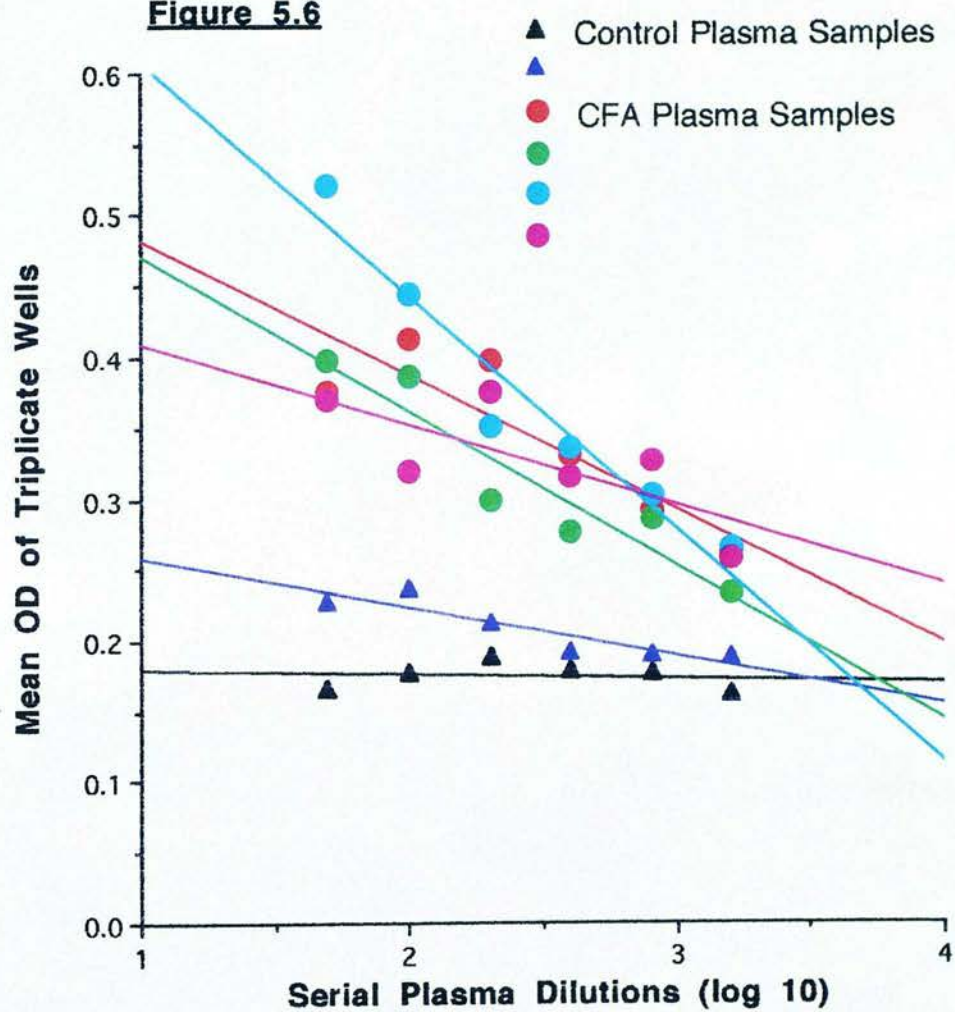


Figure 5.7

Photomicrograph of cryostat section of CFA stained with control rabbit IgG (100µg/ml) using the ABC alkaline phosphatase method described in section 2.8.4.1. No evidence of positive staining is identified on the section (Original magnification X 400).

Figure 5.8

Photomicrograph of cryostat section of CFA stained with immune rabbit IgG (100µg/ml) using the ABC alkaline phosphatase method described in section 2.8.4.1. Prominent staining of alveolar epithelial lining cells can be observed. The staining has a linear pattern in association with the flat type I epithelial cells (A) and a cytoplasmic distribution in the type II epithelial cells (B). (Original magnification X 200)

Figure 5.7

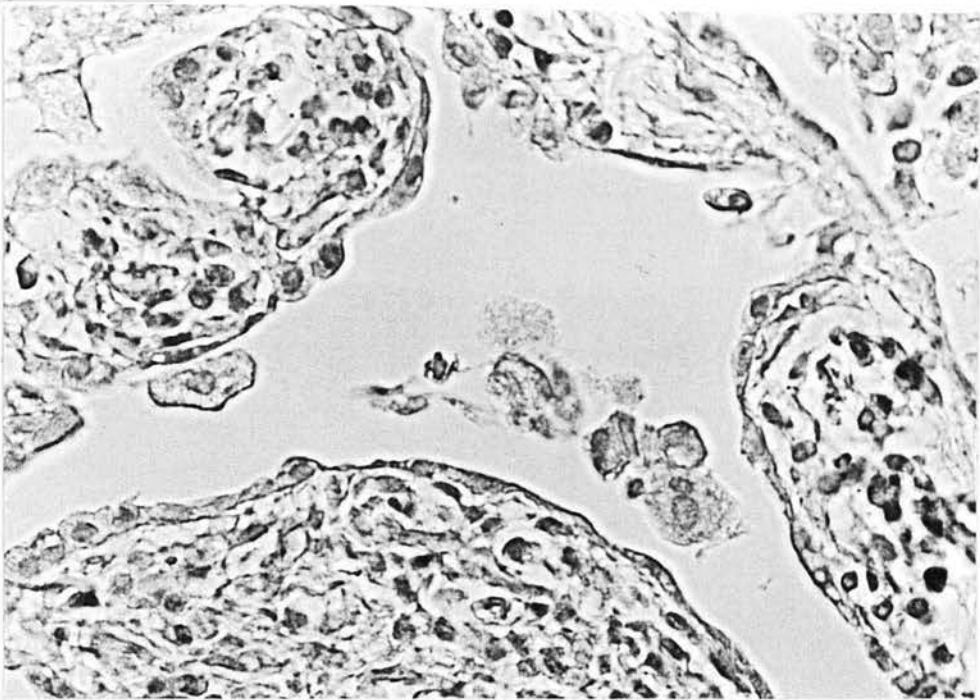


Figure 5.8

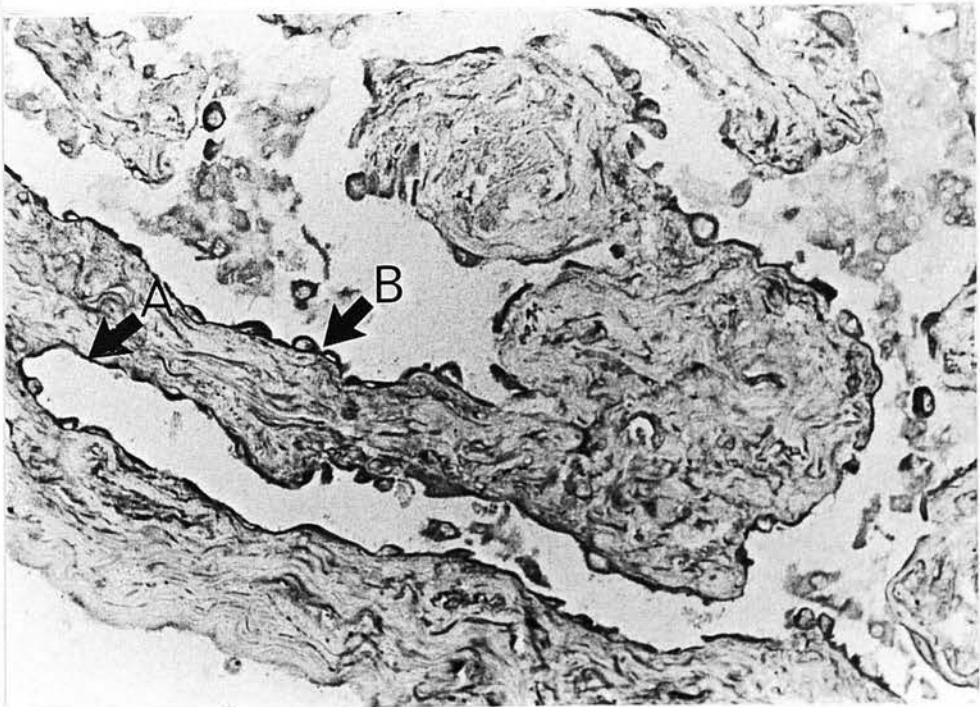


Figure 5.9

Photomicrograph of cryostat section of CFA stained with immune rabbit IgG (100 μ g/ml) using the ABC alkaline phosphatase method described in section 2.8.4.1. Staining of the ciliated surface of bronchial epithelial cells is identified (arrow). No staining of the main part of the cell is seen and the appearances suggest the possibility of antigen trapping in the cilia or overlying mucus layer. No similar positivity was evident in the parallel sections stained with the control rabbit IgG. (original magnification X400)

Figure 5.10

Photomicrograph of cryostat section of CFA stained with immune rabbit IgG (100 μ g/ml) using the ABC alkaline phosphatase method described in section 2.8.4.1. In addition to alveolar epithelial cell staining focal cytoplasmic staining of alveolar macrophages was noted (arrow) suggesting possible uptake of the antigen by these cells. (original magnification X200)

Figure 5.9

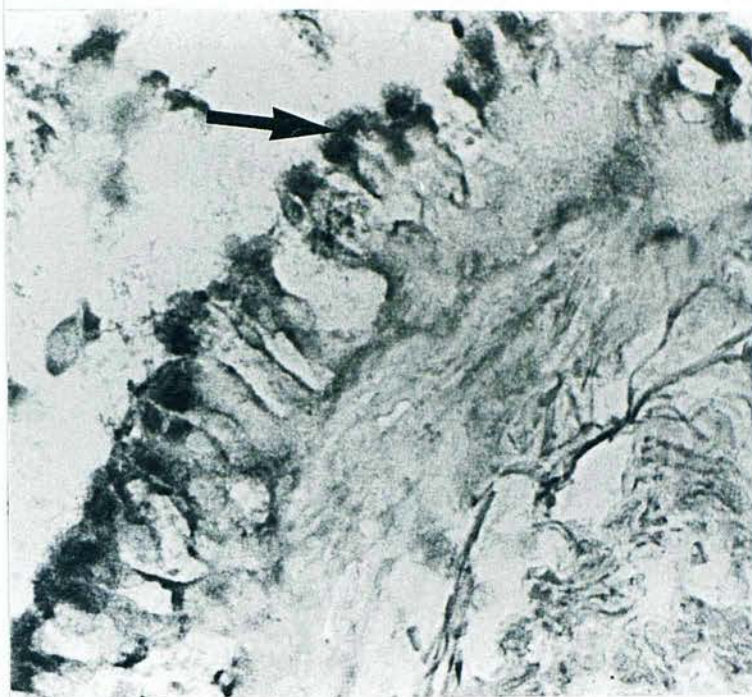


Figure 5.10

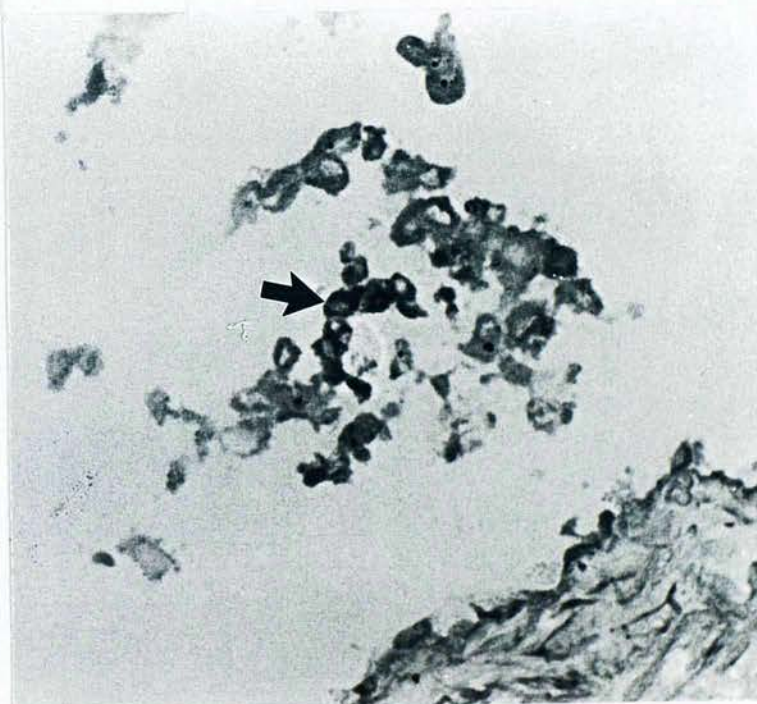


Figure 5.11

Photomicrograph of cryostat section of sarcoid lung tissue stained with (a) control rabbit IgG or (b) immune rabbit IgG (both 100 μ g/ml) using the ABC alkaline phosphatase method described in section 2.8.4.1. Faint cytoplasmic staining can be observed with the control IgG in some of the giant cells (G) within the granulomas the significance of which is uncertain. In contrast, however, sections stained with the immune IgG show prominent staining of alveolar epithelial lining cells (E), particularly immediately adjacent to the granulomas, and some alveolar macrophages (M). The giant cells (G) also show much stronger positive staining than with the control IgG. These appearance suggest that the antigen is expressed by alveolar epithelial cells in tissue lung from sarcoid patients and is compatible with the Western blotting results presented in Chapter 3. The significance of the staining of the giant cells and granulomatous macrophages is less certain given the presence of some positivity with the control IgG. (Original magnification of both figures X100)

Figure 5.11

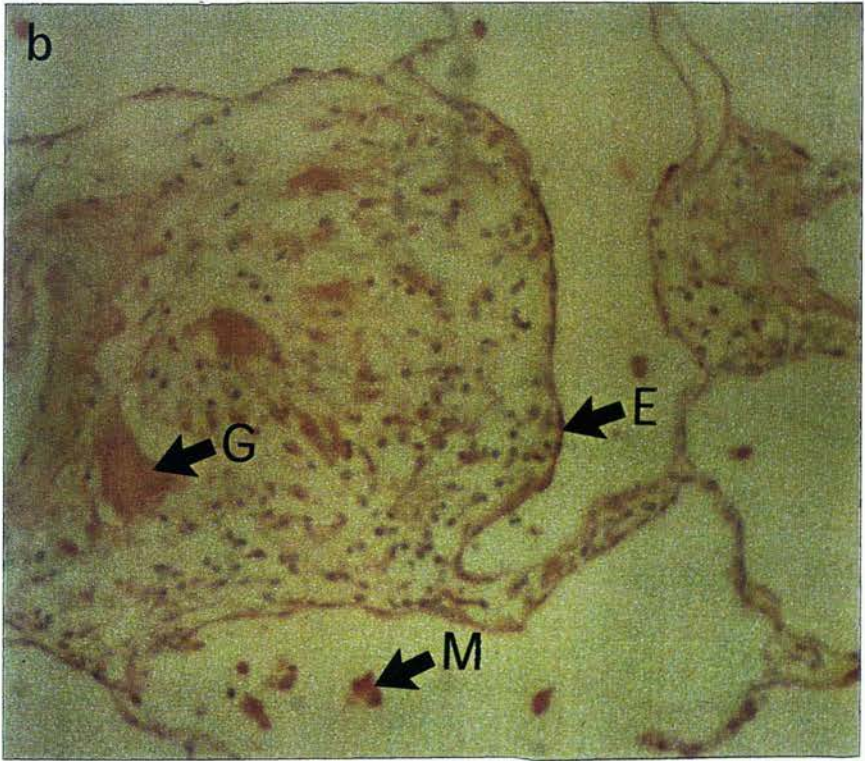
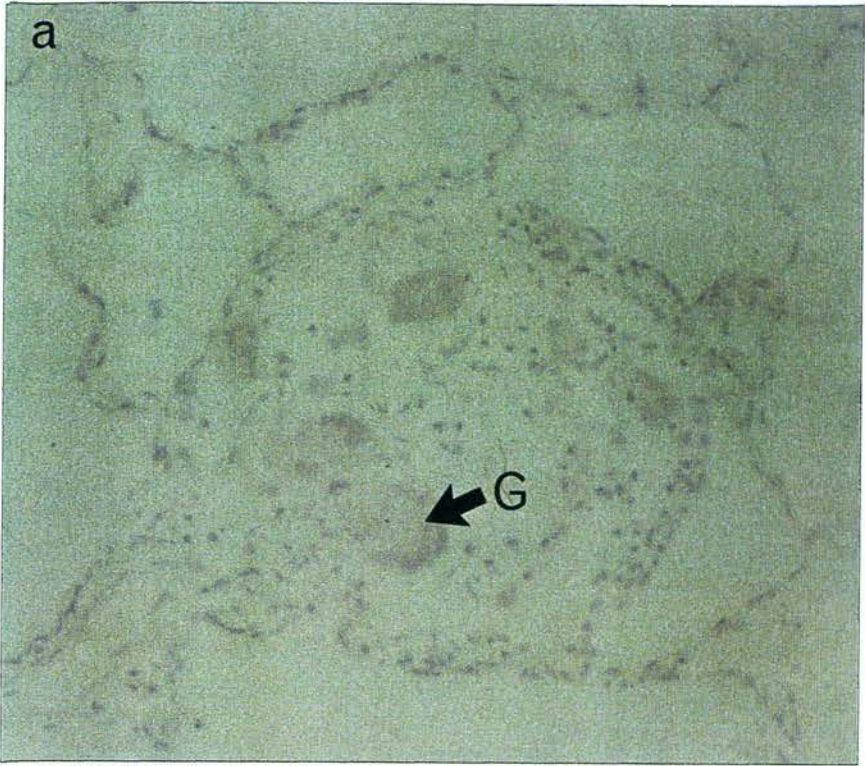


Figure 5.12

Photomicrograph of cryostat section of control, non-ILD, lung tissue stained with (a) control rabbit IgG or (b) immune rabbit IgG (both 100µg/ml) using the ABC alkaline phosphatase method described in section 2.8.4.1. No evidence of positive staining was observed with the control rabbit IgG but focal weak linear positivity was noted on the alveolar surface with the immune rabbit IgG (arrow). These appearances suggested that the antigen was present in the control lung tissue associated with the alveolar epithelial lining cells but to a lesser extent than in the ILD cases examined. (Original magnification: X 400)

Figure 5.12

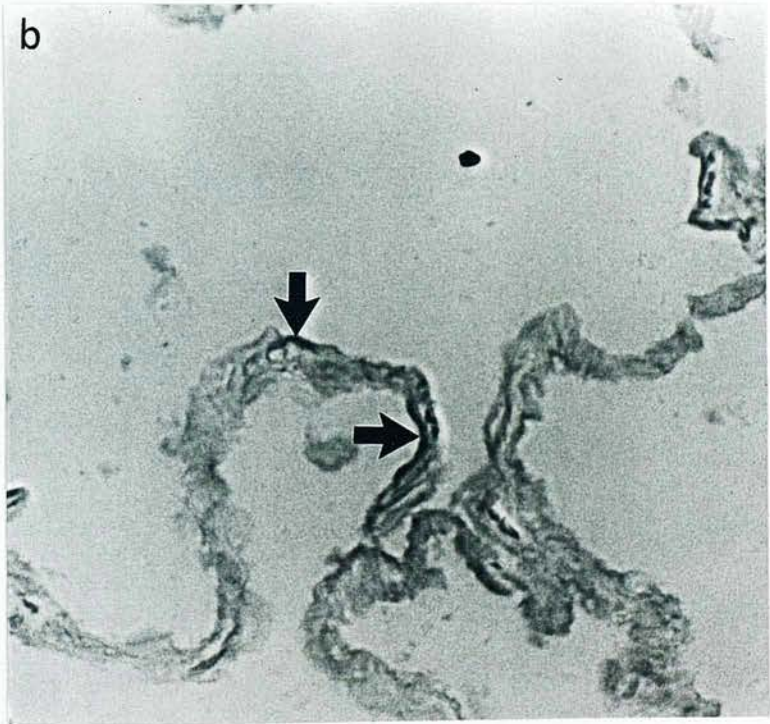
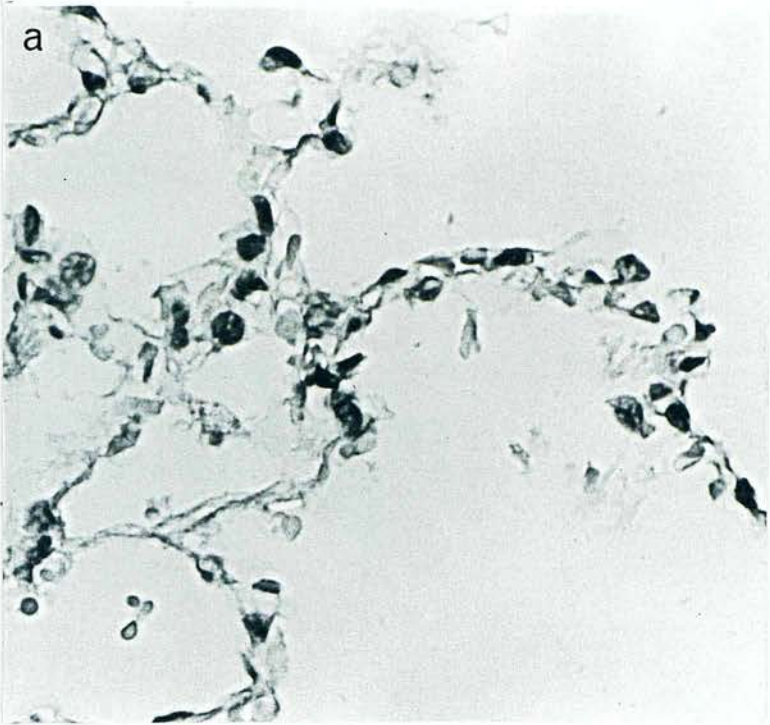


Figure 5.13

Photomicrograph of cytospin preparations of A549 cells stained with (a) control rabbit IgG or (b) immune rabbit IgG (both 100 μ g/ml) using the ABC alkaline phosphatase method described in section 2.8.4.1. Cytoplasmic staining of the A549 cells can be seen with the immune IgG but not the control. (Original magnification X 200)

Figure 5.13

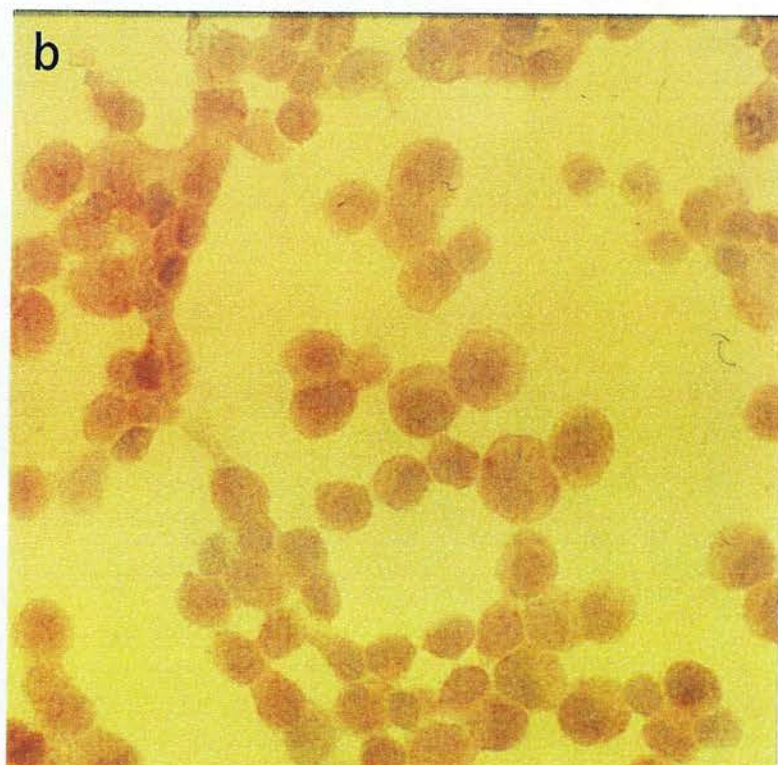
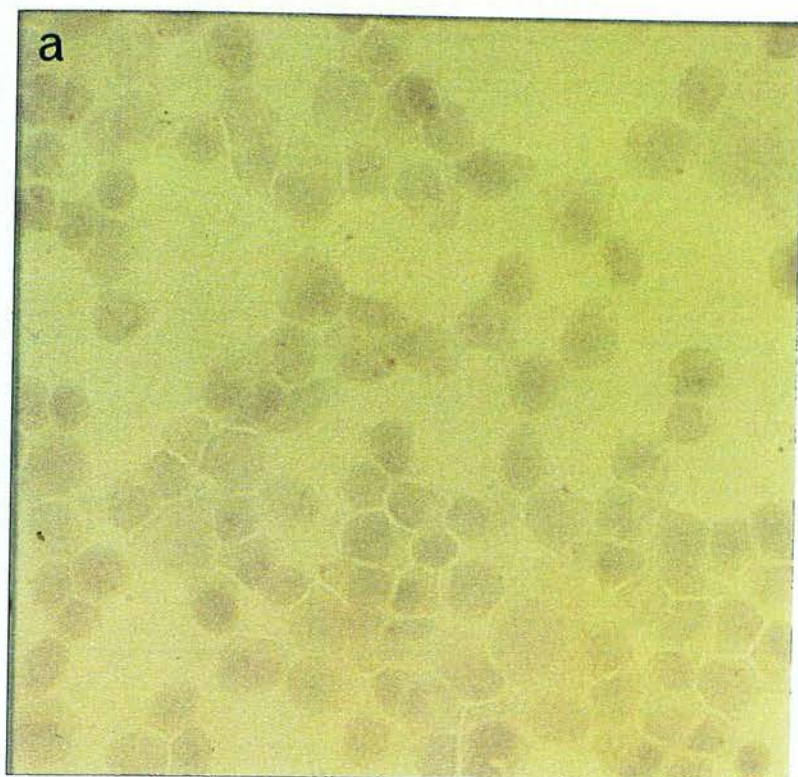


Figure 5.14

Photomicrographs of cryostat sections of (a) skin, (b) lymph node and (c) kidney stained immune rabbit IgG (100µg/ml) using the ABC alkaline phosphatase method described in section 2.8.4.1. No evidence of positive staining was detected in any of the sections examined. (Original magification, all X100)

Figure 5.14

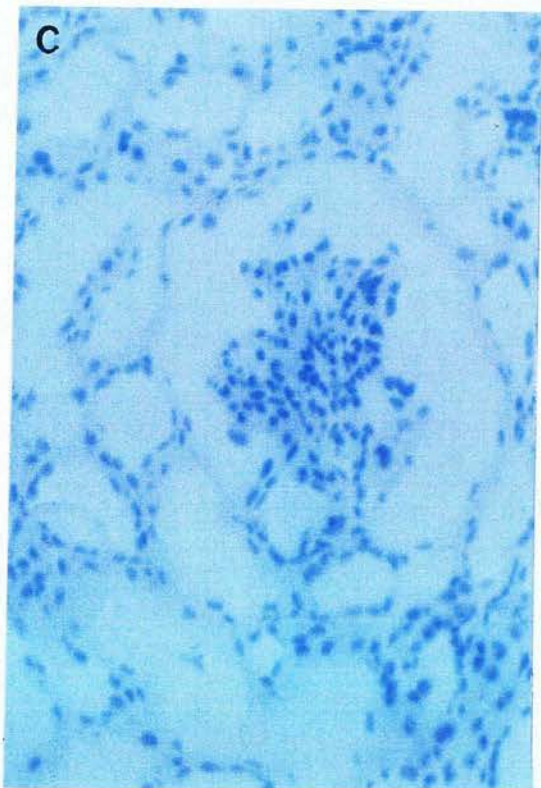
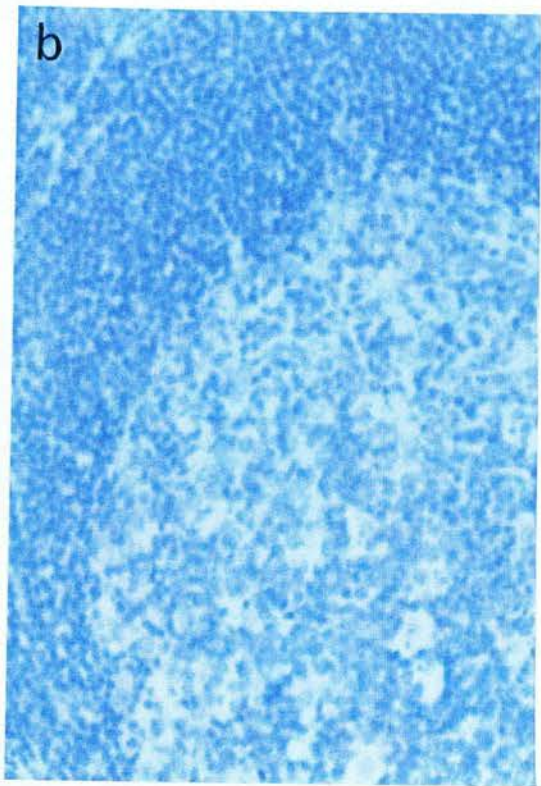
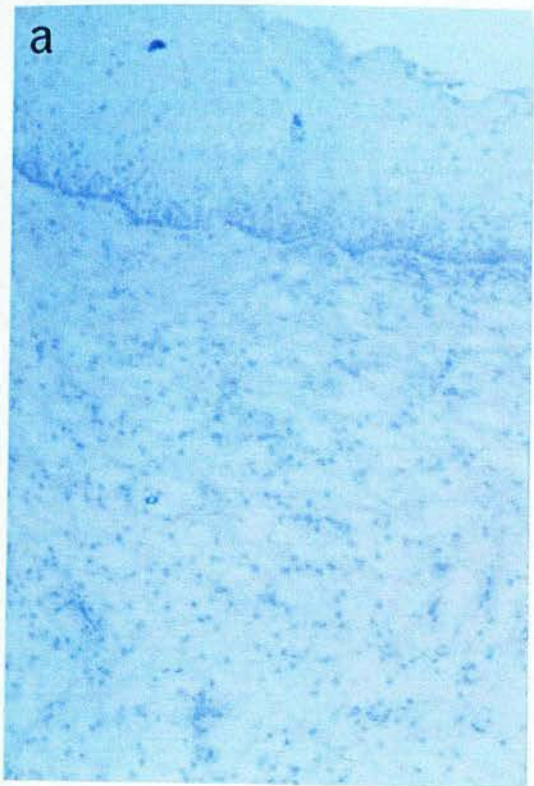


Figure 5.15

Illustration of results obtained by flow cytometry using fresh and permeabilised A549 cells obtained as described in section 2.9.1. Parallel populations of cells were stained using an indirect immunofluorescence technique following incubation with: (A) PBS (as a negative control), (B) control rabbit IgG, (C) vimentin (as a positive control for permeabilisation) and (D) immune rabbit IgG. The histograms show the results obtained for both cell population stained with each reagent as the intensity of fluorescence against frequency. The percentage of cells staining positively (see table below) are defined as those lying to the right of the cursor.

	Fresh A549 Cells	Permeabilised A549 Cells
A Negative Control	3.45%	0.57%
B Control Rabbit IgG	7.59%	4.71%
C Vimentin	12.55%	80.63%
D Immune Rabbit IgG	23.29%	66.31%

The results shown and the histogram profiles suggest that the antigen has an intracellular location in the A549 cells. While a small percentage of cells may show surface expression this may be the result of non-specific staining of non-viable cells. These results are consistent with the immunohistochemical results shown earlier for A549 cells (Fig. 5.13) and the hyperplastic type II cells in the tissue sections (Fig. 5.8) which also indicated a cytoplasmic distribution for the antigen.

Figure 5.15

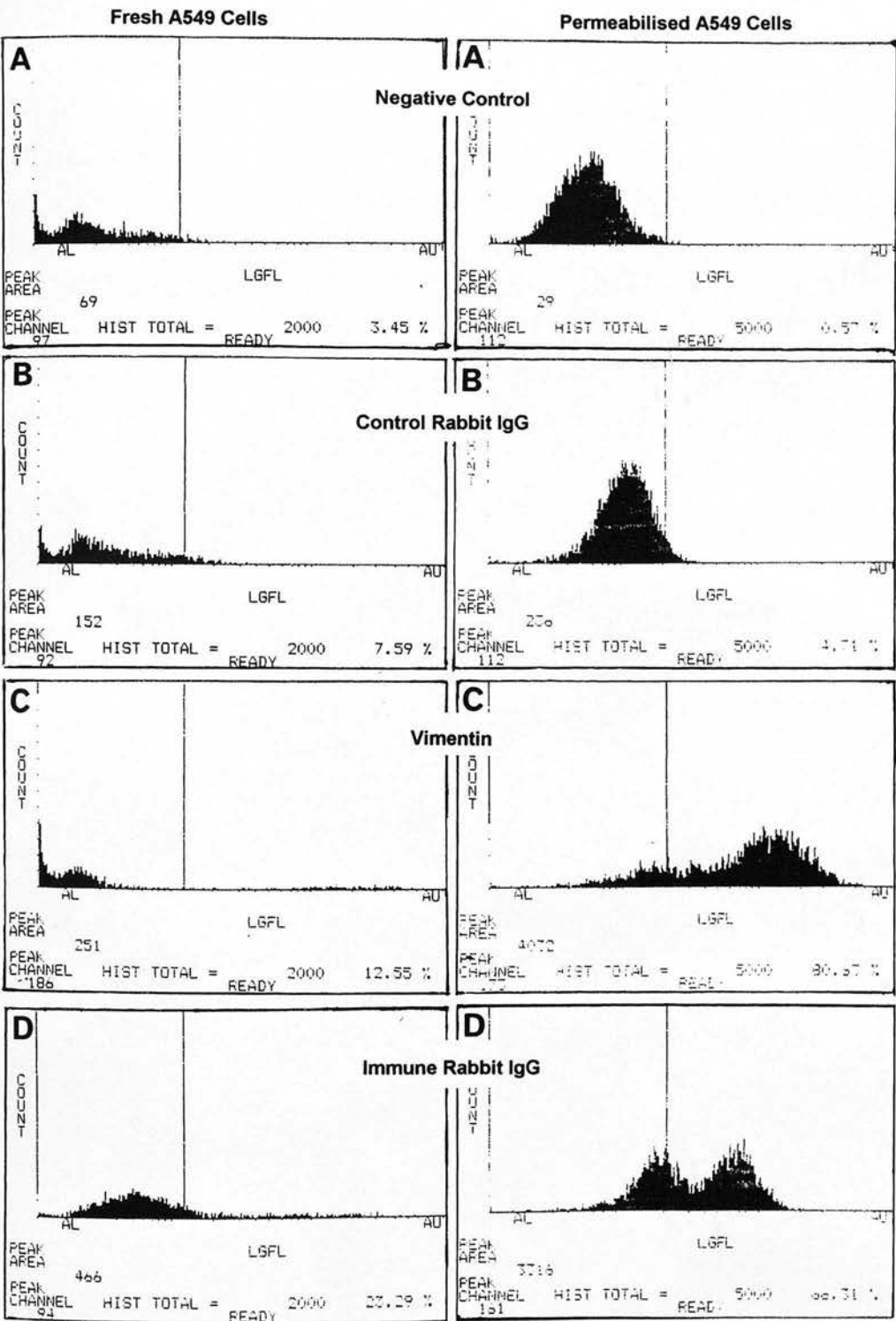
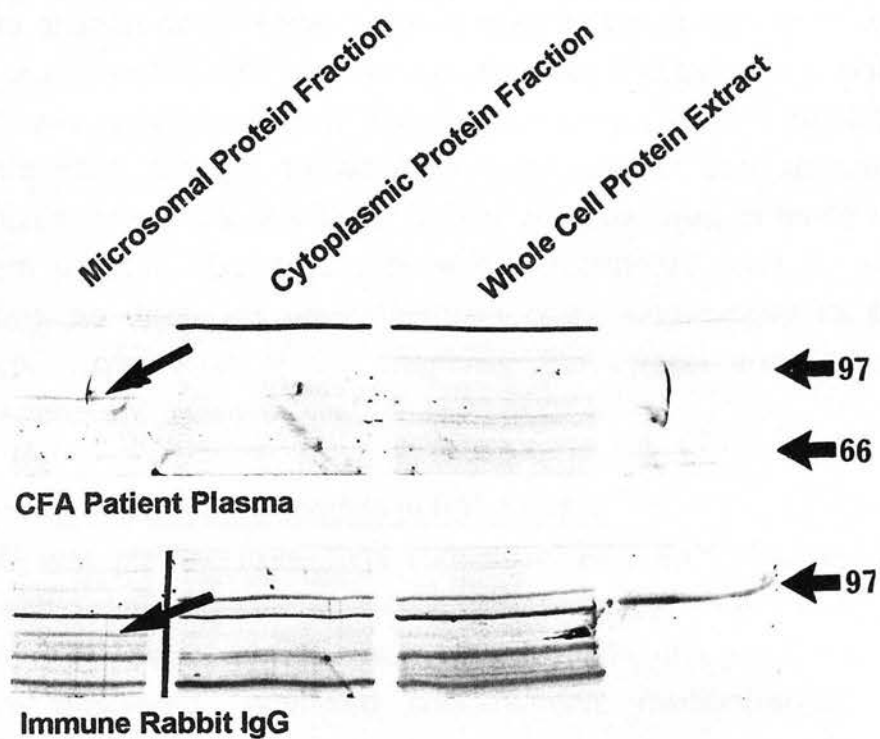


Figure 5.16

Illustration of results obtained by Western blotting using CFA patient plasma and immune rabbit IgG on microsomal and cytoplasmic proteins derived from A549 cells as described in section 2.3.2.2. The blot obtained with the whole cell extract is also shown for comparison. The molecular weight markers are indicated on the right. In both blots the 70-90 kDa antigen was detected principally in the microsomal protein fraction (arrow) with a possible fainter band in the cytoplasmic fraction possibly representing contamination. In both cases the intensity of the band was stronger in the microsomal fraction than the whole cell fraction (equal concentrations of protein being added to all 3 tracks) suggesting relative concentration of the antigen had been achieved using this technique. The bands obtained with the whole cell extract were fainter than those demonstrated previously (Fig. 3.1 and Fig. 5.1) as the amount of protein loaded onto the tracks was less so that standardisation could be achieved with the low protein yield from the differential extraction technique.

Figure 5.16



CHAPTER 6.

Studies with Bronchoalveolar Lavage Samples.

It is recognised that immune complexes may be detected in BAL samples from patients with CFA (Hunninghake 1981, Dall'Aglio 1988). In addition the results previously obtained in Chapter 5 had suggested that the 70-90 kDa antigen may be free in the lung either on its own or in complex where it was detectable in alveolar macrophages and the villi of bronchial epithelial cells in tissue sections. Experiments were therefore conducted to ascertain if either the IgG autoantibody or the antigen were detectable in BAL samples from patients with CFA. The samples used in these studies came from patients who were less well characterised than in previous experiments as they had not undergone open lung biopsy to provide histological confirmation of the diagnosis. On clinical and radiological grounds each were, however, thought to have CFA.

6.1 Detection of IgG Autoantibodies in BAL Fluid.

IgG was purified from 20ml aliquots of BAL fluid obtained from 6 patients with a clinical diagnosis of CFA as described in section 2.2.5.2. Due to the nature of the samples, which were all very dilute, only small amounts of IgG were recovered [estimated concentration: median(range) mg/ml: 0.054(0.036-0.184)].

Western blotting experiments were performed as described in section 2.4 and 2.5 using pooled protein samples derived from CFA lung biopsies in order to determine whether antibody recognising the 70-90kDa antigen was present in the BAL. The purified IgG samples were used neat in order to maximise the probability of a successful detection but despite this no bands were detected at any position on the blot other than with the control antibody, vimentin.

6.2 Detection of Auto-antigen in BAL Fluid.

20 ml aliquots of BAL from 6 patients with a clinical diagnosis of CFA were obtained and the protein content concentrated as described in section 2.2.5.3. The resultant material was run on 7.5% SDS gels under reducing conditions (section 2.4) and Western blotting (section 2.5) was performed against each sample with immune and control rabbit IgG diluted 1:10 in buffer. A sample of CFA lung protein extract was included in each experiment to act as a positive control. The results showed that the immune

rabbit IgG, but not the control, detected antigen in the 70-90 kDa weight range in 2 of the 6 cases (Fig. 6.1).

6.3 Immunohistochemical Detection of Auto-antigen in BAL Macrophages.

Cytospin preparation of BAL cells were produced as described in section 2.5.1 from 6 patients with clinically suspected CFA. The cells were stained with either buffer alone, control rabbit IgG or immune rabbit IgG using an ABC-alkaline phosphatase method (section 2.8.4.1). Positive staining of the alveolar macrophages was obtained with both the control and immune IgG as well as buffer alone indicating non-specific reactivity between the cells and the biotinylated swine anti-rabbit immunoglobulin used in the detection system. This persisted despite extensive attempts at blocking with normal swine serum, bovine serum albumin and the inclusion of Tween 20 in the buffer, thus precluding any conclusions to be drawn.

6.4 Comment.

The failure to identify any evidence of the autoantibody in the BAL samples was perhaps not surprising given the small amounts of total IgG that could be isolated. The IgG present in the airspaces is undoubtedly significantly diluted during the process of lavage and it seems likely that larger sample volumes would be required before sufficient IgG could be obtained to reliably prove or exclude the presence of the autoantibody in BAL samples.

The results do, however, suggest that the antigen is present in BAL fluid at least in some patients. The reason that 4 of the samples were negative is unclear. This may reflect the fact that the antigen is being detected as part of an immune complex and these are certainly recognised only to be detectable in a variable percentage of patients (see section 1.10). Alternatively it may relate to differential concentrations of protein obtained from the samples and loaded onto the gel. Due to the small volumes obtained following concentration the protein content of the samples were not estimated. Thus, while the negative results may be unreliable, the presence of a positive result in two of the cases confirms that the auto-antigen may be present in the airspaces at least in some individuals.

The results presented in Chapter 5 suggested that the auto-antigen may be present in some alveolar macrophages and this would certainly be predicted if the antigen exists in the airspace as an immune complex.

Immunohistochemical staining of CFA lung biopsies has previously indicated the presence of C1q on the alveolar surface of the lung and in the cytoplasm of alveolar macrophages supporting this notion (Wallace 1994). Unfortunately immunohistochemistry with the immune rabbit sera was unsuccessful in demonstrating this on lavaged cells because of high levels of non-specific background staining. Alveolar macrophages are difficult to perform reliable immunohistochemistry on because of the high numbers of Fc receptors expressed on their surface. This technical problem does, however, appear to be more pronounced in lavaged cells than with cells stained *in situ* within lung sections suggesting that the process of lavage may further activate the cells or in some other way alter their cell surface.

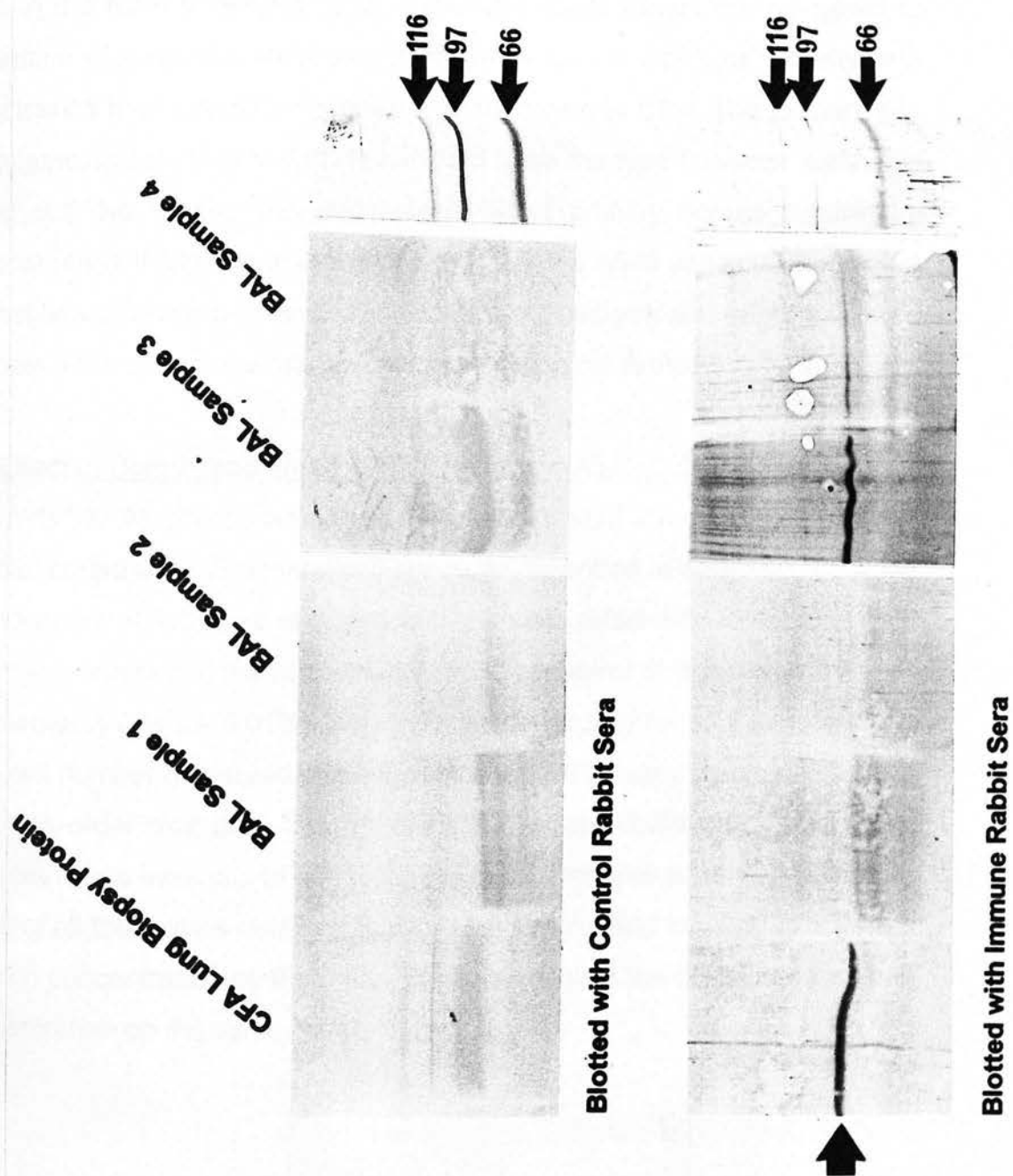
6.5 Conclusion.

The 70-90 kDa antigen was demonstrated to be present in BAL samples from some patients with CFA but the IgG autoantibody could not be demonstrated.

Figure 6.1

Illustration of representative results obtained using Western blotting to detect the 70-90 kDa antigen in bronchoalveolar lavage (BAL) fluid from 4 patients clinically suspected of having CFA. Protein extracted from CFA lung biopsy tissue was included in one of the tracks to act as a control. Blotting was performed with control and immune rabbit sera and developed using a biotinylated swine anti-rabbit immunoglobulin as described in section 2.5. The molecular weight markers are indicated on the right. The immune but not the control rabbit serum detected antigen in the 70-90 kDa weight range in the lane containing CFA lung protein extracts in a manner to that which has been previously demonstrated (Chapter 5). The immune sera also detected antigen of the same molecular weight in 2 of the BAL samples indicating the presence of antigen in the airspaces of at least some patients with CFA.

Figure 6.1



CHAPTER 7.

In-vitro Biological Activity of Immune Rabbit Serum.

The previously described studies demonstrated the presence of an IgG autoantibody to a presumptive autoantigen associated with alveolar epithelial cells but gave no indication of any possible role that they might have in the pathogenesis of CFA. Experiments were therefore designed to determine whether the antibodies might have in vitro biological activity with significance to the disease processes encountered in CFA. The primary site of immunological injury in CFA is believed to be the type I alveolar epithelial lining cell. No access was available to either primary human cultures or appropriate cell lines so it was decided to use the A549 cell line as a model epithelial cell which expressed the putative autoantigen and might therefore indicate if the antibodies had any potential biological activity.

7.1 Effect of Control and Immune Rabbit Sera on A549 Cultures.

1x10⁵ A549 cells were plated into each well of 96 well plates in RPMI supplemented with 10% foetal calf serum as described in section 2.3.1. After 24 hours the media was removed and the cells refed with RPMI with 10% foetal calf serum and the addition of a range of control or immune rabbit sera supplements (10% - 0.01%). The cells were cultured for 72 hours and the final cell number measured indirectly using the MTT assay (section 2.3.3).

In order that data from different test plates could be combined for analysis it was essential to normalise the data. This was done by individually dividing all the values obtained for both the control and immune rabbit sera at each concentration by the mean OD obtained with the control sera at that concentration on the same plate.

Normalised Value =

$$\frac{\text{OD value for each individual well at x\% control or immune rabbit sera on the plate}}{\text{mean OD value obtained for all wells treated with x\% normal rabbit serum on the same plate}}$$

This resulted in the control wells (those treated with the control sera) having a geometric mean of 1.0 at each concentration tested on every plate. The normalised data from 3 separate experiments were then combined and the geometric mean and standard error of the combined data was plotted as the cell viability index against concentration of rabbit serum added to the wells. Statistical analyses were performed with a Mann-Whitney Test to compare the cell viability index obtained with the immune sera and the control sera at each concentration.

The results (Fig. 7.1) revealed a significant dose dependent reduction in the cell viability index with the immune rabbit sera in comparison to the control sera over a range of concentrations from 0.03% - 0.3%. At higher concentrations the effect appeared to be progressively lost. Examination of the raw data suggested that this was due to a reduction in the OD values obtained in the control wells rather than a progressive loss of biological activity in the wells treated with the immune sera. This suggests that the apparent loss of effect at higher concentrations of rabbit serum may be artifactual, representing progressive non-specific toxicity in the presence of high total serum concentrations.

7.2 Effect of Control and Immune Rabbit Sera on L2P6 Human Pulmonary Fibroblast Cultures.

In order to ascertain whether the above results were specific for a cell line expressing the antigen recognised by the immune rabbit serum identical

experiments were conducted with a pulmonary fibroblast cell line which did not express the antigen. No significant difference in the numbers of cells obtained after 72 hours in culture was observed at any of the concentrations employed (Fig. 7.2).

7.3 Confirmation of an IgG Mediated Effect of Immune Rabbit Serum Effect on A549 Cultures.

In order to ascertain if the effect of the immune sera on A549 cells was due to immunoglobulin acting via complement fixation and not due to a differential growth factor concentration identical experiments were conducted using IgG purified from control and immune rabbit sera as a source of immunoglobulin in the presence and absence of complement.

Cells were cultured in RPMI supplemented with 10% foetal calf serum for 24 hours and then refed with fresh RPMI supplemented with either 10% fresh rabbit serum or 10% rabbit serum that had been heat inactivated at 56°C for 30 minutes (Coombs 1961). Control or immune IgG was added to wells at a dose of either 30µg, 10µg, 3µg, or 1µg in a standard volume of PBS per well. The cells were then cultured for 72 hours and an MTT assay performed. The results of 2 separate sets of experiments were normalised and the cell viability index calculated as above.

In the presence of fresh rabbit sera the immune IgG showed evidence of a significant dose dependent reduction in the cell viability index compared to the control IgG (Fig 7.3) although this appeared to be progressively lost at higher doses. The explanation for this again appeared to relate to non-specific toxicity in the control wells. No such dose related reduction in the cell viability index was observed in the presence of the heat inactivated serum except at the highest concentration tested (Fig 7.4). These results suggested that the effect on the A549 cells of the immune sera was IgG mediated and significantly enhanced by heat sensitive factor, or factors, present in fresh serum which would seem most likely to represent complement

7.4 Effect of Control and CFA Patient Derived IgG on A549 Cultures.

Identical experiments were performed to those described above using IgG pooled from 4 CFA patients and 4 healthy controls in the presence of fresh rabbit sera to provide complement activity. The results were normalised and the cell viability index calculated using data from 2 separate experiments (Fig. 7.5). A significant reduction in the cell viability index was observed with the CFA patient IgG at a dose of 30 μ g ($p < 0.05$) but not with the other doses tested.

7.5 Comment.

The presence of the immune rabbit serum in culture appears to result in a decrease in the cell viability index after 72 hours at concentrations of 0.03 - 0.3 %. At higher concentrations the effect was progressively lost, possibly due to non-specific toxicity. The effect appears to be antigen dependant in that no significant reduction in the cell viability index was evident with the fibroblast cell line. Further experiments with a range of other cell lines may have helped to clarify the specificity of this effect.

In order to determine whether the observed effect was a non-specific feature of the immune sera or IgG mediated further experiments were conducted. IgG was purified from both the control and immune rabbit sera and tested at the same concentrations on the A549 cells in the presence of fresh or heat inactivated rabbit serum. The action of the immune IgG was similar to that of the whole serum but appeared to require the presence of a heat sensitive serum element. This observation suggested that the effect might be IgG/complement mediated although it is recognised that heat inactivation does not specifically remove complement and may result in the denaturation of other serum proteins. Further investigation would be required utilising purified complement to clarify this point.

The reason for the observed reduction in the cell viability index is uncertain especially given the evidence that the antigen is predominantly

cytoplasmic in nature. The possibility that a subset of cells show low level surface expression has previously been considered (Chapter 5) but not convincingly demonstrated. Further studies utilising control immune sera and other cell lines not expressing the 70-90 kDa antigen will be required to elucidate the specificity of the reaction and to examine possible mechanisms of action of the immune rabbit IgG.

7.6 Conclusion.

The rabbit sera directed against the putative 70-90 kDa autoantigen may have an antigen dependent effect on A549 cells in vitro. The mechanisms which result in the reduction in the cell viability index are not apparent and require further investigation.

Figure 7.1

Illustration of results obtained comparing the effect of control and immune rabbit sera on A549 cells. The data shown is that accumulated from 3 separate experiments normalised around the results obtained with the control rabbit sera as described in section 7.1. The data is presented as geometric mean and standard error (n=12) and analyses were performed using a Mann-Whitney Test. A dose dependant reduction in the cell viability index was observed in the wells treated with immune rabbit sera compared to those treated with the control sera. This appeared to be progressively lost at higher concentrations of added rabbit serum and may relate to a non-specific general reduction in the cell viability index in the wells treated with the control sera rather than loss of specific activity at higher concentrations of the immune sera.

Figure 7.1

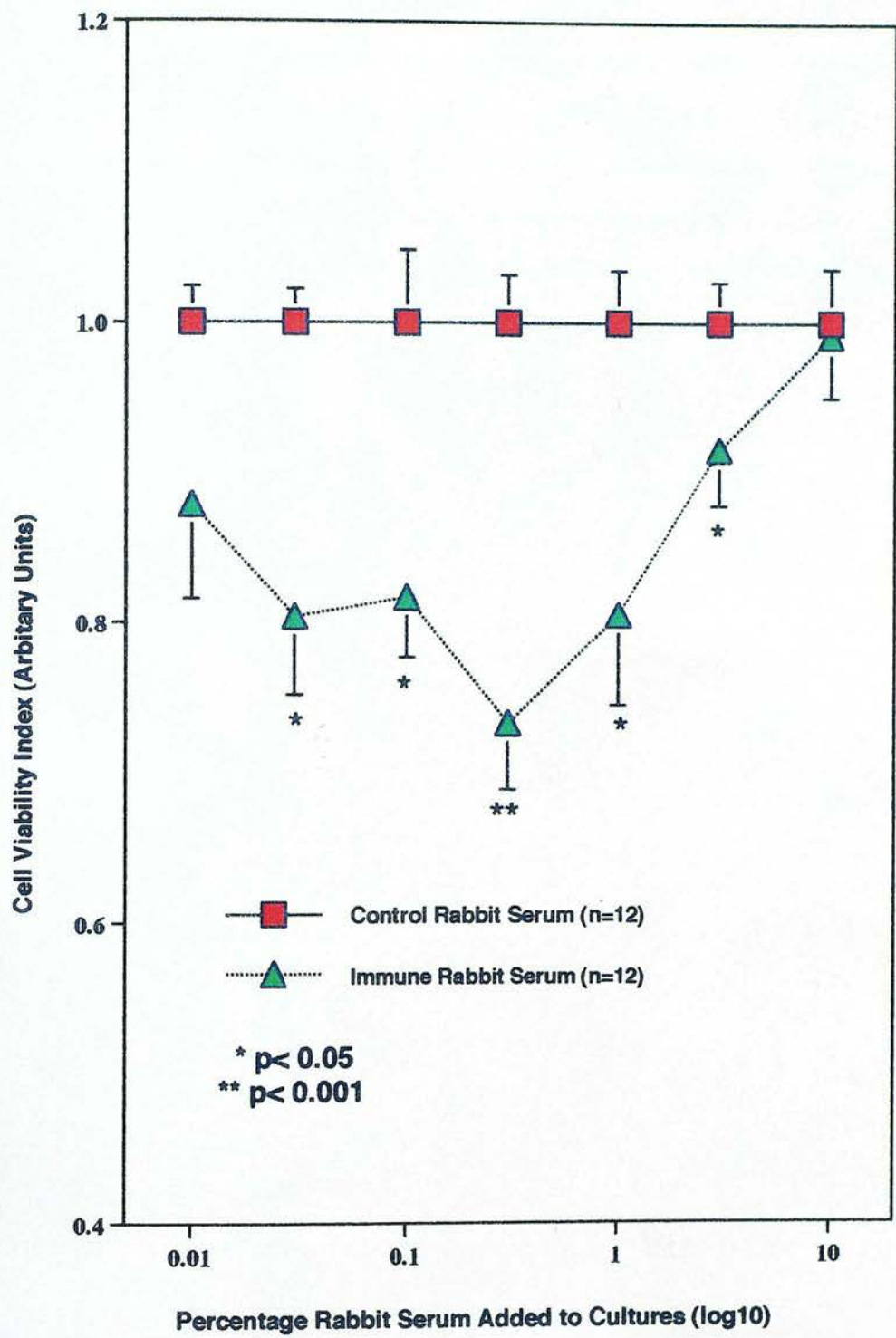


Table 7.2

Illustration of results obtained comparing the effect of control and immune rabbit sera on L2P6 pulmonary fibroblasts. The data shown is that accumulated from 2 separate experiments normalised around the results obtained with the control rabbit sera as described in section 7.1. The data is presented as geometric mean and standard error (n=12) and analyses were performed using a Mann-Whitney Test. No significant difference in the cell viability index between the wells treated with the control and immune sera was identified at any of the concentrations tested.

Figure 7.2

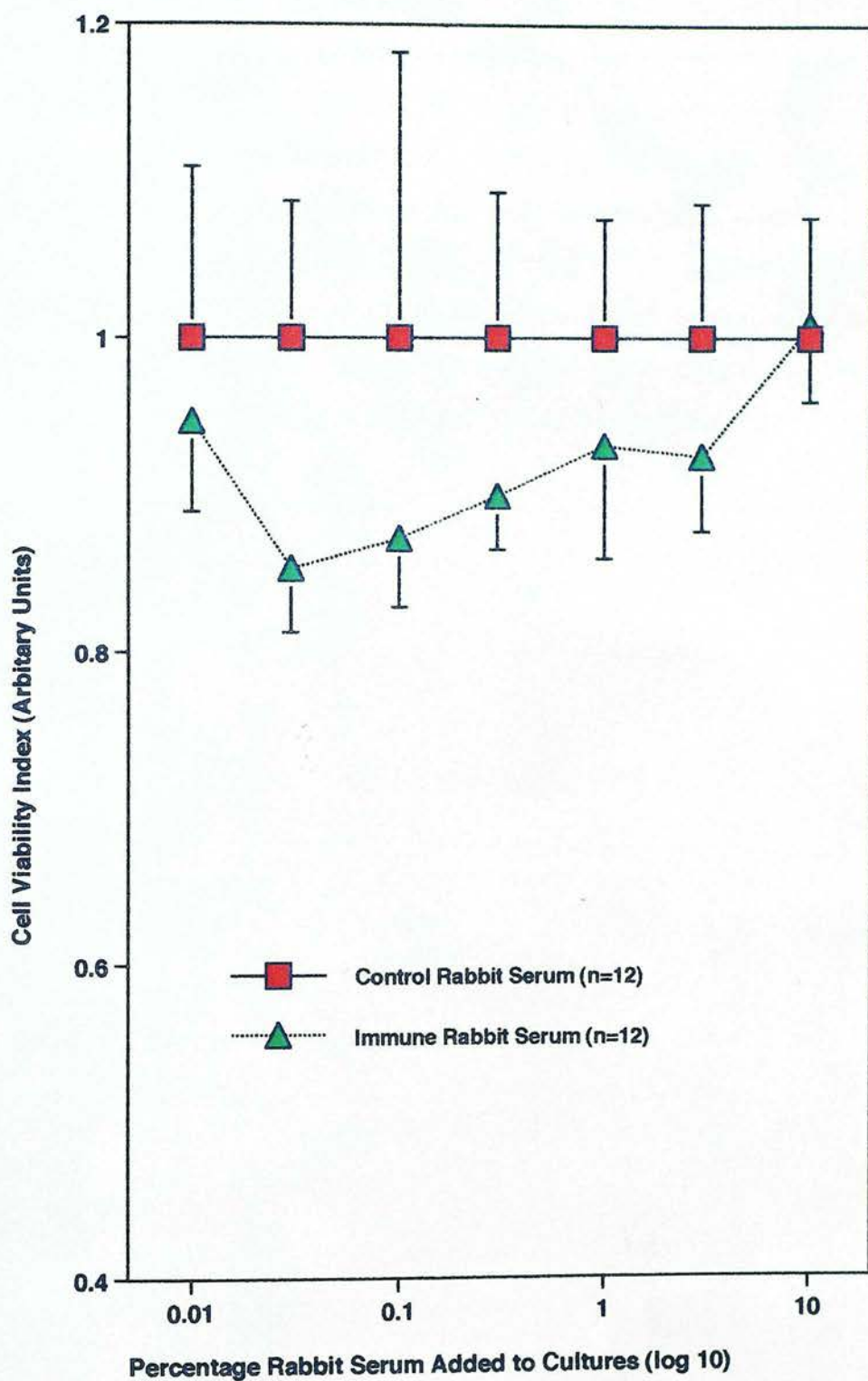


Figure 7.3

Illustration of results obtained comparing the effect of control and immune rabbit IgG on A549 cells in the presence of fresh rabbit serum. The data shown is that accumulated from 2 separate experiments normalised around the results obtained with the control rabbit sera as described in section 7.1. The data is presented as geometric mean and standard error (n=8) and analyses were performed using a Mann-Whitney Test. The results revealed a significant dose dependant reduction in the cell viability index in the wells containing the immune IgG compared to the control with a pattern similar to that observed in the experiments using the sera (Figure 7.1). This effect, again, appeared to be partially lost at higher concentrations of rabbit IgG possibly reflecting non-specific toxic effects in the cultures.

Figure 7.3

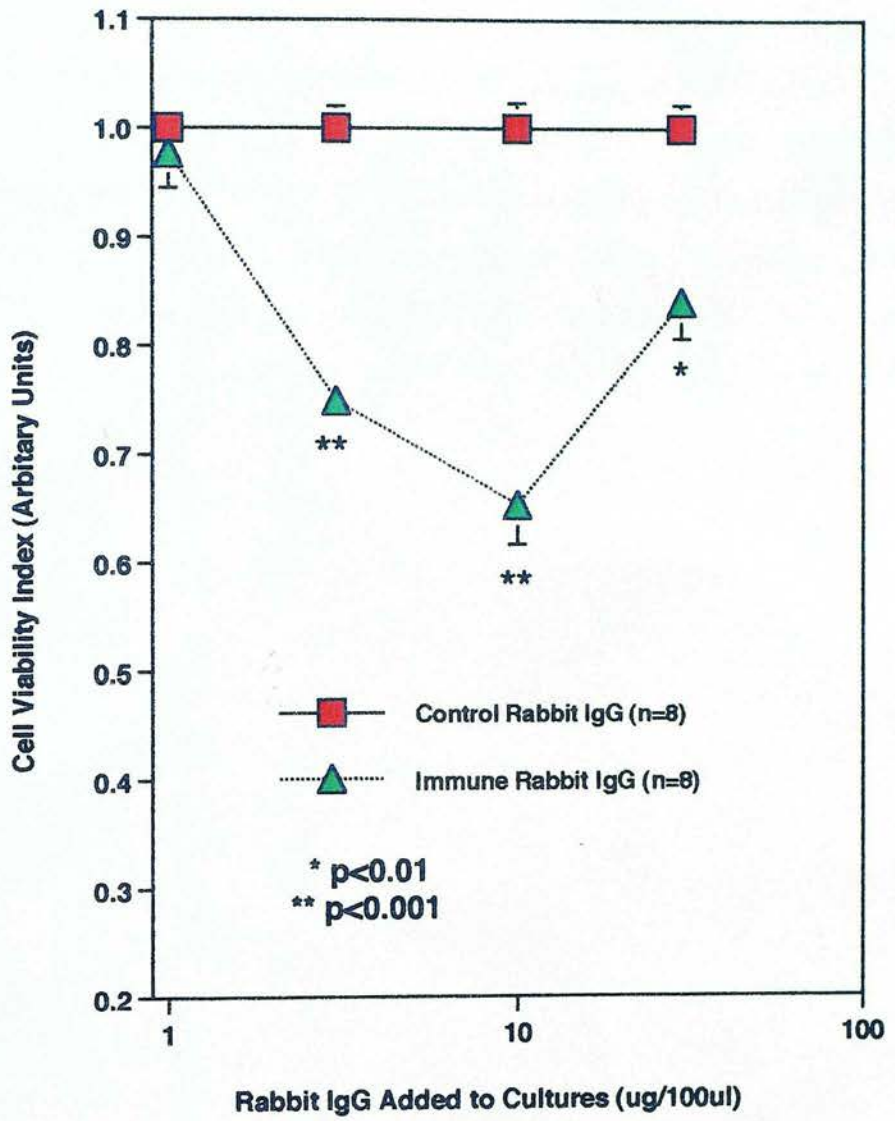


Figure 7.4

Illustration of results obtained comparing the effect of control and immune rabbit IgG on A549 cells in the presence of heat inactivated rabbit serum. The data shown is that accumulated from 2 separate experiments normalised around the results obtained with the control rabbit sera as described in section 7.1. The data is presented as geometric mean and standard error (n=8) and analyses were performed using a Mann-Whitney Test. In contrast to the results obtained in parallel experiments conducted with fresh rabbit serum (Figure 7.3) no significant reduction in the cell viability index was observed except at the highest concentration tested (30 μ g per well). These results suggested that the reduction in cell viability brought about by the immune rabbit sera was IgG mediated and enhanced by the presence of heat sensitive serum factors.

Figure 7.4

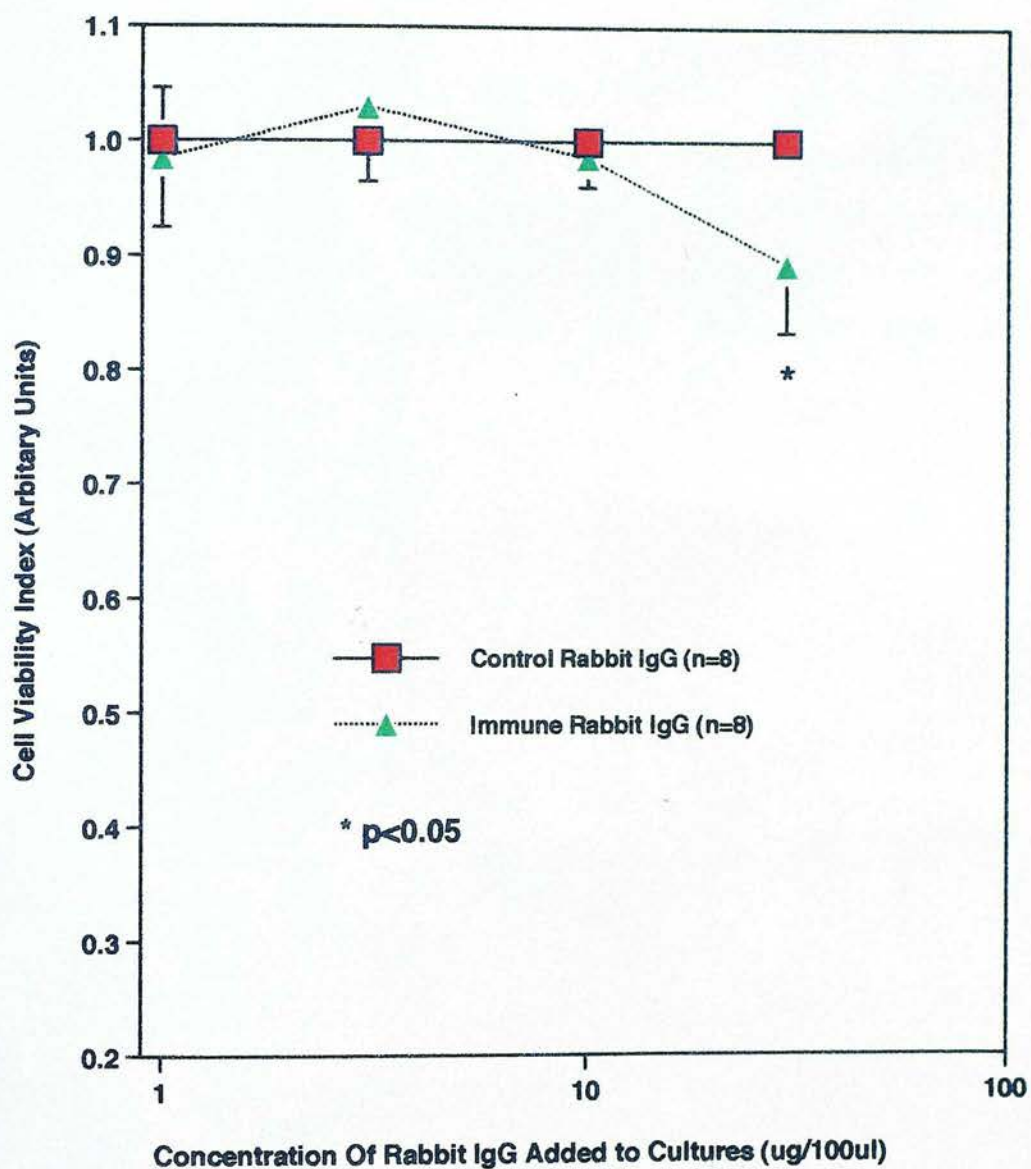
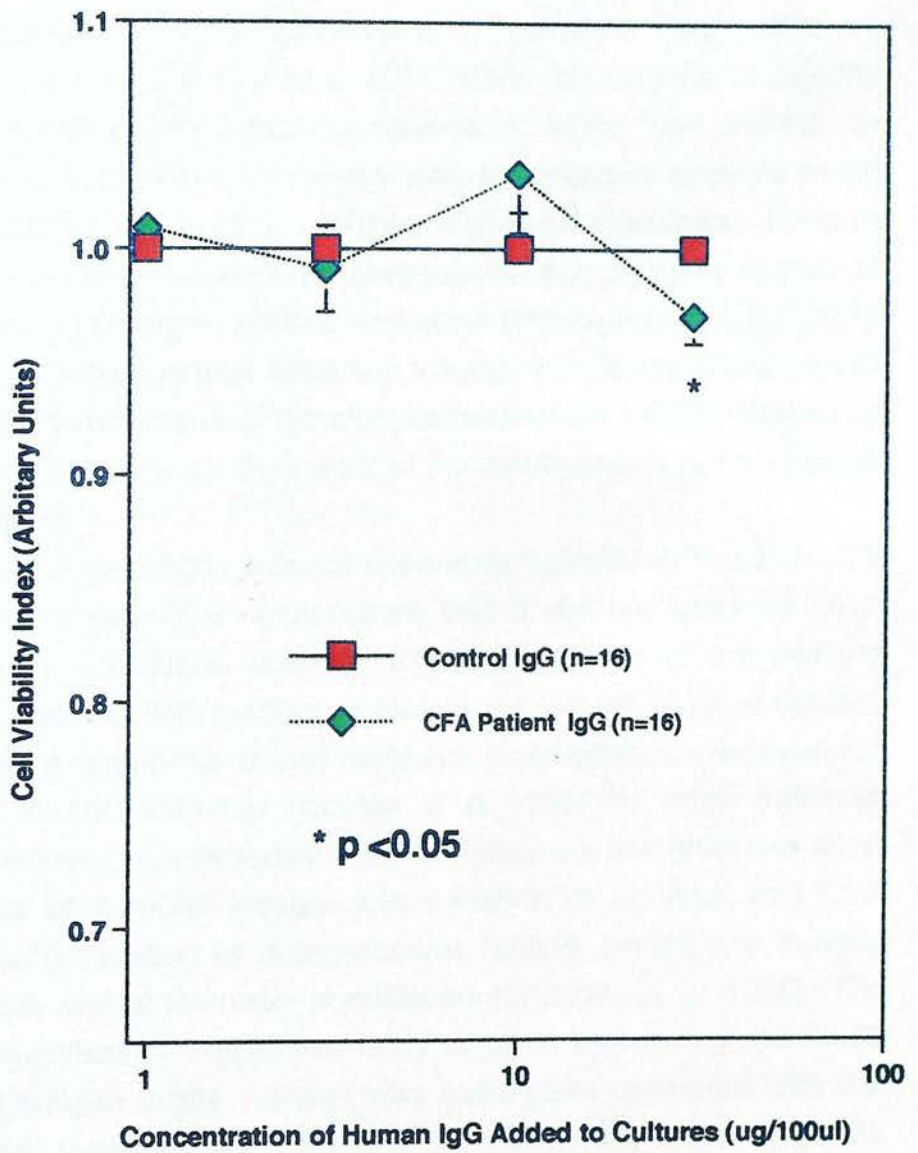


Figure 7.5

Illustration of results obtained comparing the effect of IgG purified from CFA patients and controls on A549 cells in the presence of fresh rabbit serum. The data shown is that accumulated from 2 separate experiments normalised around the results obtained with the control rabbit sera as described in section 7.1. The data is presented as geometric mean and standard error (n=16) and analyses were performed using a Mann-Whitney Test. No significant reduction in the cell viability index was observed in comparing the CFA IgG with the control IgG except at the highest concentration tested (30µg per well). The apparent lack of a significant reduction in the cell viability index with the patient IgG compared to the control IgG except at this level may reflect the relatively low titre of specific autoantibody that would be present in the purified IgG.

Figure 7.5



8.1 Significance of Autoantibodies in CFA.

The hypothesis examined in this thesis was that the development of autoantibodies to a lung antigen might occur in patients with CFA and that this might have a role in the pathogenesis of the clinico-pathological entity. The results presented confirm for the first time that patients with CFA have a high incidence (95%) of circulating IgG autoantibodies to a 70-90 kDa lung specific antigen associated with alveolar epithelial lining cells.

Autoantibodies with no pathological significance are, however, recognised to occur at low titre to a wide variety of antigens in healthy individuals (Avrameas 1991) the significance of which are unclear. In addition the frequency with which these may be detected appears to be increased in patients with evidence of chronic immune stimulation. Patients with CFA show evidence of immune dysregulation and certainly appear to frequently develop non-organ specific antibodies (Hance and Crystal 1983). This suggests a rather general tendency towards the development of self reactive immune responses in CFA making assessment of the significance of the autoantibodies detected with respect to the pathogenesis of the disease difficult to interpret.

While the autoantibody was not apparently detectable in the healthy controls it was necessary to demonstrate that it did not occur in other inflammatory lung conditions where similar dysregulation of the immune system occurs. Patients with pulmonary sarcoidosis provided a good disease control group for this purpose as they represent a pathologically well defined population of patients with ILD (Cotram *et al* 1989) on which sufficient numbers of samples could be easily obtained. They are recognised to show similar features of humoral immune dysregulation to patients with CFA resulting in the generation of autoantibodies against lymphocyte surface molecules which are of unknown significance (Spurzen *et al* 1988). The absence of a significantly higher frequency of autoantibodies to the 70-90 kDa epithelial antigen in the patients with sarcoidosis compared with the healthy controls, therefore, provided stronger evidence that the response might be a specific feature of the disease process in CFA than that obtained using the healthy controls.

The present studies do not indicate whether the antibody response arises as a consequence of epithelial injury or whether epithelial injury

results from the presence of the autoantibody. The *in vitro* results presented in Chapter 7 might suggest the latter as a possibility but *in vivo* studies of patients who have been exposed to a defined episode of lung injury with epithelial damage will be necessary to confirm this. One potential group of patients where this may be possible are those identified as being at risk of developing ARDS following trauma or major sepsis. Initial baseline samples could be used as controls and samples obtained from patients who survived an episode of ARDS could be tested to see if a response to this antigen had been generated. Failure to detect the autoantibody in these patients, who pathologically show significant epithelial injury with type II cell hyperplasia (Hasleton 1983), would strongly suggest that the response was not simply secondary to epithelial cell injury but that it had a more specific role in CFA. Such studies are now on-going in order to answer this question more fully.

8.2 The nature of the auto-antigen.

The nature of the antigen detected by the autoantibody remains unclear but the possibility that it is viral in origin seems unlikely given that it can be detected at low levels in control lung tissue and in the A549 cell line which is not known to be virally infected or transformed (Lieber *et al* 1976). The results suggest that it is likely to be endogenous with probable up-regulation in CFA. It has been clearly demonstrated to have a predominantly cytoplasmic distribution in A549 cells and in the type II cells in tissue sections. The distribution of the antigen associated with the type I cells could not be assessed by light microscopy as these cells are not visible using this technique. The pattern of staining with the immune rabbit IgG confirmed that antigen was associated with them but whether this had a cytoplasmic or membranous distribution or even was present in the overlying layer of fluid which lines the alveolar spaces was not decipherable. The question of the location of the antigen associated with the type I cells must therefore await study using immuno-gold electronmicroscopy.

Circumstantial evidence suggests that the antigen may be a secretory product of type II cells as it appears to be associated with the microsomal fraction of the A549 cells and it is recognised that the primary biological role of the type II epithelial cell in the lung is the synthesis of proteins which are secreted into the airspaces (Hawgood 1991). This hypothesis would suggest that the antigen may in fact be type II specific with an intraluminal

distribution following secretion into the alveolar lining fluid accounting for the observed pattern of staining associated with the type I cells.

One obvious candidate group of antigens which would fit this suggestion are those present in surfactant. Surfactant is composed principally of phospholipids but also contains a variety of proteins most of which are derived from plasma. In addition, however, three specific surfactant apoproteins are recognised and designated as SP-A, SP-B, and SP-C (reviewed Hawgood 1991). These apoproteins are produced by type II epithelial cells and are secreted into the surfactant monolayer on the alveolar surface. The molecular weight of these proteins as determined by SDS-PAGE under reducing conditions is 28-36 kDa, 9 kDa and 4 kDa respectively, although SP-B and SP-C may exist in pre-protein forms of 43 kDa and 21 kDa. The fully processed form of SP-A which is secreted by the cell is recognised to consist of an oligomer of 18 identical monomeric protein units arranged in a partial double helix assembly. Once secreted the oligomeric form interacts with phospholipids in a temperature dependent fashion although the significance of and regulation of these protein-lipid interactions is unclear. Thus SP-A in its functional state is likely to have a molecular weight range in excess of 500 kDa. Less is known about the biochemistry of SP-B and SP-C which together account for only 1-2% of the surfactant mass.

While the idea that the antigen might be a surfactant apoprotein is attractive, given the demonstrated distribution and apparent pulmonary specificity of the antigen, the results obtained do not fully support this. The molecular weight of the auto-antigen detected was in the order of 70-90 kDa as detected by SDS-PAGE under reducing conditions. This does not match the known molecular weights of any of the 3 apoprotein peptides although an SP-A dimer could fall into this weight range (56-72 kDa). This, however, seems unlikely to be the explanation as under reducing conditions intensely staining bands would have been identified in the monomeric position (28-36 kDa). In order to exclude this, however, it would be necessary to obtain monoclonal antibodies to SP-A which worked on Western blotting and confirm that the 70-90 kDa antigen was not detected.

Initially when it was realised that the autoantigen was up-regulated in ILD tissue it was hypothesised that the antigen might be a molecule which was immunologically active and expressed on the infiltrating cells. When it was shown that the antigen was confined to the alveolar epithelial

component of the lung and could not be detected in reactive tonsillar tissue this hypothesis was discarded. It is, however, recognised that alveolar epithelial cells may express immunologically active molecules such as HLA-DR (personal observation) and ICAM-1 (Mulligan *et al* 1993, Holt 1993) in CFA and although these are not specific to epithelial cells the possibility remains that some form of regulatory molecule specific to these cells is being induced.

An alternative explanation for the upregulation of the antigen in CFA is that the relative number of type II cells is greater than that of the controls. In CFA type II cells are more prominent and thus more antigen may be available in the tissue for extraction simply due to a relative increase in their number rather than an increase in antigen expression *per se*. Such an explanation would also be compatible with the concept of the antigen being type II cell specific.

Although the nature of the autoantigen remains unknown, theoretically if it is of any pathological significance in the disease then it must be lung specific as most (>90%) patients with CFA do not have evidence of systemic disease (Hunninghake and Fauci 1979). Alveolar epithelial lining cells undoubtedly play a highly specialised role in permitting gas exchange and it therefore seems likely that they will have functional molecules associated with them that are not present in other cell types.

8.3 Pathogenetic significance of autoantibodies in CFA.

While the Western blotting data suggests that the development of the autoantibodies might not be simply a reflection of immune dysregulation it did not show evidence of any pathological role in the disease process. The *in vitro* cell culture studies presented in this thesis, however, indicated an antigen dependent effect on A549 cells which was IgG and complement mediated. It is not clear at this stage what the mechanism responsible for the reduction in the cell number obtained is. Although these experiments were conducted with a type II cell line, as no type I cell line or primary cultures were available, the immunohistochemical data suggests that the antigen is at least associated with type I cells and therefore by analogy similar effects might be observed *in vivo*. These autoantibodies might therefore form the basis of the immune complexes described in CFA and have a role, as discussed in Chapter 1.10, in the pathogenesis of epithelial injury and activation of macrophages.

The role of autoantibodies in the pathogenesis of organ specific autoimmune diseases has been much debated (Avrameas 1991). It has long been held that tissue damage in such conditions as Hashimoto's Thyroiditis is due to cellular mechanisms and that the associated development of autoantibodies to thyroglobulin has no pathological role (Charreire 1989). Recent studies in rabbits with autoimmune thyroiditis have, however, indicated that complement depletion substantially reduces the degree of damage and the authors suggested that while the disease may be initiated by cell mediated immune processes the development of autoantibodies acted to enhance the disease process *via* complement fixation (Inoue *et al* 1993). The presence of autoantibodies and immune complexes may, therefore, have more significance in the pathogenesis of inflammatory disease in terms of producing tissue damage than has been generally accepted.

In order to examine the role of the autoantibodies in the pathogenesis of CFA it will be necessary to try and show evidence of lung injury in animal models in which a similar humoral response has been induced. Such experiments have in part already been started as the rabbit immunised to produce the polyclonal serum described in Chapter 5 developed circulating antibodies to the putative autoantigen. The animal currently remains well and has continued to produce the antibody following further booster injections of antigen with no outward sign of lung disease. This might suggest that the antibody has no pathological role but the question of cross species reactivity of the antibody has not been addressed and neither has the animal's lungs been examined histologically. Another theoretical explanation for the failure of the rabbit to develop pulmonary disease might be that the autoantibody is only capable of playing a role in maintaining the process once epithelial damage has been initiated by other means. This would seem more analogous to the situation in CFA where the initiating process remains unknown but seems unlikely to be the autoantibody itself.

The possibility that the type II epithelial cell population might also represent a target in the pathogenetic processes resulting in CFA does not appear to have been considered. These cells are believed to respond to type I cell injury rather than being a primary focus of the disease process. The demonstration, however, that these cells express the antigen and that the antibody has an *in vitro* effect raises the possibility that the type II cells may also represent an immunological target. While, ultrastructurally, type II

epithelial cell injury does not appear to be a feature of CFA (Corrin *et al* 1985) the results obtained, in Chapter 7, show only a reduction in total A549 cell number, compared with controls, after 72 hours in culture. This could, theoretically, have resulted from a reduction in the proliferation rate of these cells rather than cell death. This suggests a possible new antibody mediated mechanism which could contribute to the *in vivo* fibrosis observed in CFA.

As discussed earlier (section 1.10.6) Adamson *et al* have shown that restoration of epithelial integrity by proliferating type II cells may be important in the regulation of fibroblast function (Adamson *et al* 1988 and 1990). Anything which delays this process may therefore promote fibrosis while stimuli such as KGF which enhance type II proliferation may have a protective effect (Panos 1994 Personal communication). If the effect of the autoantibody was therefore to delay, or inhibit, re-epithelialisation of damaged alveolar walls by reducing the proliferative capacity of the type II cells then dysregulated fibroblast activity might ensue *in vivo*. Such non-lethal effects on the cell might also explain the pattern of type II alveolar epithelial cell atypia observed in CFA.

It is also recognised that these atypical type II cells are capable of producing a wide range of pro-inflammatory and fibrogenic agents (section 1.10.7) and that they may be capable of interacting with fibroblasts and macrophages *via* signals regulated by autocrine feedback loops. It has been shown that surface stimulation of A549 cells in culture by agents which are known to be fibrogenic *in vivo*, but not inert substances, result in release of the chemokine MIP-2 (Driscoll 1994 Personal communication). The signalling mechanisms responsible for this differential effect are unknown, but one possibility remains that fibrogenic agents are fibrogenic in nature because they exert non-lethal effects on cells which inert substances do not. Thus an antibody directed against a type II cell antigen might result in similar non-lethal effects promoting interactions with the inflammatory and mesenchymal cell populations resulting in the perpetuation of the immunological and fibrogenic processes.

The results of antibody mediated effects on type II epithelial lining cells would therefore be potentially additive according to this hypothesis. The cells would be stimulated to produce inflammatory and fibrogenic factors while the inhibitory effect of re-epithelialisation on fibroblast function would be delayed. Such a scenario would be consistent with the pathological association of proliferating fibroblasts with overlying atypical hyperplastic

type II cells (see section 1.4). The nature of any such effect is unknown but the absence of ultrastructural evidence of type II cell necrosis in CFA (Corrin *et al* 1985) suggests that it may be more specific than simply cell membrane damage.

This model would dictate that epithelial injury *per se* was not sufficient to allow the development of the clinico-pathological syndrome of CFA and that the development of the autoantibodies to the type II cell antigen was required before this was likely to occur. This suggestion would thus fit with the epidemiological data which has suggested diverse aetiological triggers and the requirement for a genetic component. Injury to the lung which did not result in the generation of these autoantibodies, either because of the type of injury induced or because of the genetic make-up of the individual, would not result in the development of the pattern of disease recognised as CFA. Other individuals exposed to the same agent might respond differently and develop CFA.

Despite this demonstration of autoantibodies to an autoantigen in patients with CFA no explanation for the odd distribution of the disease (see Chapter 1) has been obtained. The distribution of the antigen as assessed with the immune rabbit IgG appears to include areas of lung which do not show evidence of disease although assessment was difficult due to the limitations of the frozen section morphology. If the disease process was solely regulated by the autoantibody then a more uniform episode of acute lung injury would be anticipated rather than the spatially and temporally separated foci which are observed.

What is apparent is that whatever processes operate to produce the clinico-pathological syndrome recognised as CFA the mechanisms involved must provide an explanation for the pattern of disease observed. Currently no such explanation has been put forward despite the belief that the process is immunologically mediated and therefore presumably antigen driven. The possibility that some form of 'permissive event' may occur allowing immunological reaction to antigen in a focal manner raises the possibility of further disease regulating mechanisms within the lung that have not previously been appreciated.

8.4 Clinical Perspectives.

Despite scepticism of the role that autoantibodies may play in the pathogenesis of inflammatory diseases their presence has proved useful in

the diagnosis and monitoring of a variety of immunologically mediated diseases including some affecting the lung. This includes such conditions as Goodpasture's Syndrome (Wheeler *et al* 1988) and Wegener's Granulomatosis (Gross *et al* 1993) where the detection of autoantibodies to glomerular basement membrane and neutrophil cytoplasmic antigens respectively have an established clinical role in the diagnosis and monitoring of disease activity.

Thus while the role of the autoantibodies in the pathogenesis of CFA may be speculative their presence raises the possibility of a diagnostic or prognostic serological test. In the samples used in Chapter 3 testing for the presence of the autoantibody had a diagnostic sensitivity of 95% and a specificity of 76% for CFA. The use of Western blotting, however, to routinely screen plasma samples for the presence of autoantibodies is cumbersome and is further limited in that measurement of titre is not realistically possible.

In order for the antibody to be detected and quantitatively measured it will be necessary to develop an enzyme linked immunosorption assay (ELISA). The data presented in Chapter 5 indicates that the autoantibody can be detected in plasma samples using an antigen capture technique with crude lysates of A549 cells as a source of the antigen. This observation suggests that the development of an ELISA to detect and measure the autoantibody titre may be a real possibility. This development might provide the means of monitoring the disease activity in patients with CFA in the simple reproducible manner that is urgently required to allow better monitoring of disease activity and the development of new therapeutic strategies.

8.5 Cryptogenic Fibrosing Alveolitis: Autoimmune disease?

As discussed in Chapter 1 a considerable amount of evidence exists which suggests that CFA has an immunological pathogenesis. The work presented in this thesis suggests that an autoantibody to alveolar epithelial lining cells, believed to be the site of immunological damage in CFA (Corrin *et al* 1985), is generated. This raises the question of whether CFA should now be regarded as an autoimmune condition.

Rose and Bona (1993) recently reviewed criteria for defining diseases as autoimmune in nature. He argued that direct proof could be obtained if evidence of identical disease could be detected in an individual receiving a

transfusion of sera containing the autoantibody or autoantigen specific T cells. The latter is clearly not possible in humans but the former is recognised to occur physiologically across the placenta and accounts for neonatal myasthenia gravis, Graves' disease and polychondritis.

Indirect evidence can be obtained by producing an animal model of the disease by immunisation with the putative human autoantigen and subsequent transfer of T cells to allogeneic animals. Such experiments inducing anti-myelin basic protein CD4 positive T cells have suggested that this may be the pathogenetic mechanism responsible for demyelination in multiple sclerosis. The isolation of autoantibodies or self reactive T cells from the affected tissue may also constitute indirect evidence of an autoimmune process. Autoantibodies can be isolated from the kidneys of patients with SLE and Goodpasture's Syndrome while cytotoxic T cells specific for thyrocytes have been isolated from thyroid tissue in patients with Graves' Disease. Rose (1993) argues that the association of a condition with other autoimmune diseases, lymphocytic infiltration of the affected organ, statistical association with a particular MHC haplotype or aberrant expression of MHC II antigens in the affected organ or evidence of response to immunosuppression constitutes merely circumstantial evidence.

These criteria are, however, applicable if a disease is to be characterised as purely autoimmune in nature. Many inflammatory conditions would appear to be complex in their pathogenesis and it seems likely may be driven by immune processes directed against multiple antigens some of which may be autoimmune in nature. Thus while the current evidence for CFA being an autoimmune disease is not strong according to the criteria set by Rose, the probability must be that there is an autoimmune component to the disease process which results in the clinico-pathological disease as it presents to physicians and pathologists.

8.6 Future Studies.

8.6.1 Identification of the autoantigen.

Identification of the autoantigen by protein purification has proved difficult given the reagents currently to hand although this might become more realistic with the development of a monoclonal antibody. Current interest is focusing on using genetic techniques to isolate the antigen from the A549 cells using a cDNA library constructed from total mRNA in *E. coli* expression vectors. The clones will then be screened using both the rabbit

antisera and CFA patient IgG to detect clones of interest which can then be sequenced. Such techniques have previously been used to identify the autoantigen in pemphigus as a cadherin (Amagai *et al* 1991).

8.6.2 Development of an ELISA.

Research on this aspect of the project is continuing with the aid of a 2 year grant from Chest Heart and Stroke Scotland to develop an ELISA. This will allow serial titres of autoantibody to be measured and related to disease activity and allow screening of large patient groups in order to assess the specificity of the autoantibody to the diagnosis of CFA.

8.6.3 Interactions of Autoantibody with Type II Alveolar Epithelial Cells.

Further study is required to ascertain the interactions of the hyperplastic epithelial cells, autoantibodies, locally produced cytokines and extracellular matrix components. The possibility that the autoantibody induces the type II epithelial cell to play a pivotal role in maintaining the inflammatory and fibrogenic processes observed in CFA is an interesting hypothesis which merits further careful consideration and study.

Appendix A

Age, Sex and Smoking Histories of Patients and Volunteers Studied.

Table 1.

CFA Patients.

Patient No.	Age	Sex	Smoking History
1*	68	M	S
2*	59	M	S
3*	62	F	NS
4	53	F	NS
5	61	M	Ex S
6	69	M	S
7	60	F	NS
8	71	M	S
9	77	F	NS
10	66	M	Ex S
11	68	M	S
12	49	M	S
13	70	F	Ex S
14	80	F	NS
15	67	F	S
16	66	M	NS
17	78	F	NS
18	63	M	S
19	62	M	Ex S
20	69	M	S
21	70	M	S
22	68	M	Ex S

* Denotes patients diagnosed without open lung biopsy

S = Current smoker, NS = Non-Smoker, Ex S = Ex-Smoker

Table 2.**Sarcoid Patients.**

Patient No.	Age	Sex	Smoking History
1	32	M	NS
2	46	F	NS
3	42	F	NS
4	38	M	NS
5	63	F	NS
6	48	M	NS
7	63	F	NS
8	36	M	S
9	28	M	NS
10	33	M	NS
11	30	M	NS
12	57	M	NS
13	38	F	Ex S
14	53	M	NS
15	35	M	NS
16*	48	F	Ex S
17	28	M	NS
18	54	F	NS
19*	46	M	NS
20*	41	F	NS
21*	45	M	NS
22*	54	F	NS

* Denotes patients diagnosed by open lung biopsy

S = Current smoker, NS = Non-Smoker, Ex S = Ex-Smoker

Table 3a.

Volunteers (Non-Smokers)

Subject No.	Age	Sex
1	30	M
2	36	M
3	32	M
4	37	F
5	45	M
6	38	M
7	42	M
8	52	F
9	49	M
10	56	F
11	48	M
12	55	F
13	43	F
14	58	F
15	29	M
16	28	F
17	28	M

Table 3b.
Volunteers (Smokers)

Subject No.	Age	Sex
1	26	M
2	36	M
3	59	M
4	33	M
5	40	M
6	22	M
7	40	M
8	57	F
9	54	F
10	51	M
11	57	F
12	44	M
13	39	F
14	26	F
15	24	M
16	37	M

Appendix B

Monoclonal (mc) antibodies and polyclonal (pc) antisera used in Western blotting experiments.

Antibody/serum	Species	Source	Catalogue / clone No.
mc anti-vimentin	mouse	DAKO UK Ltd.	M7020
mc anti-human IgG (Fc specific)	mouse	SIGMA UK Ltd.	GG5
mc anti-human IgA (Fc specific)	mouse	SIGMA UK Ltd.	GA112
mc anti-human IgM (Fc specific)	mouse	Dr K. Guy, Strathclyde University	DA127
biotinylated anti-mouse IgG	rabbit	DAKO UK Ltd.	EO464
biotinylated anti-mouse IgG	swine	DAKO UK Ltd.	EO453

Appendix C

Publications arising as a result of the work presented in this thesis and related studies.

1. WAH Wallace, SN Roberts, H Caldwell, E Thornton, AP Greening, D Lamb and SEM Howie. Circulating antibodies to lung protein(s) in patients with cryptogenic fibrosing alveolitis. Thorax 1994;**49**:218-224.
2. WAH Wallace, JA Schoffield, D Lamb and SEM Howie. Localisation of a pulmonary auto-antigen in cryptogenic fibrosing alveolitis. Thorax 1994;**49**:1139-1145.
3. WAH Wallace, SEM Howie, D Lamb and DM Salter. Tenascin immunoreactivity in cryptogenic fibrosing alveolitis. J. Path. 1995;**175**:415-420.
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5. WAH Wallace, EA Ramage, D Lamb and SEM Howie. A type 2 (Th2 like) pattern of immune response predominates in the pulmonary interstitium of patients with cryptogenic fibrosing alveolitis. Clin Exp. Immunol. 1995;**101**:436-441.

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Circulating antibodies to lung protein(s) in patients with cryptogenic fibrosing alveolitis

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Abstract

Background – It has been hypothesised that cryptogenic fibrosing alveolitis has an immunological pathogenesis mediated by T lymphocytes. It is, however, recognised that patients may show dysregulation of the humoral immune system and that the presence of large numbers of B lymphocytes in open lung biopsies may be associated with a poor prognosis. Evidence of a role for the humoral immune system in the pathogenesis of cryptogenic fibrosing alveolitis has been suggested, but attempts to demonstrate circulating immunoglobulin to antigen within the lung have been inconclusive.

Methods – Plasma samples from 22 patients with cryptogenic fibrosing alveolitis, 22 patients with sarcoidosis, and 17 healthy controls were screened by SDS-PAGE and Western blotting for the presence of autoantibodies to lung proteins derived from cryptogenic fibrosing alveolitis, sarcoid and control lung tissue, as well as four normal non-pulmonary tissues. Possible site(s) of target protein(s) within the lung tissue were identified by immunohistochemical examination using IgG purified from the plasma of six patients and two controls.

Results – Eighteen of the plasma samples from patients with cryptogenic fibrosing alveolitis had reactive IgG to lung protein(s) in the 70–90 kDa molecular weight range compared with five of 18 plasma samples from patients with sarcoidosis and one of 17 controls. Plasma from patients with cryptogenic fibrosing alveolitis recognised antigen(s) of the same molecular weight in control and sarcoid lung tissue, but not non-pulmonary tissues, with a similar frequency. Immunohistochemical staining of cryptogenic fibrosing alveolitis biopsy material using IgG purified from plasma samples from patients with cryptogenic fibrosing alveolitis, but not control samples, revealed fine linear positivity in the lung parenchyma in a pattern suggestive of reaction with alveolar lining cells. The pattern was cytoplasmic/membranous and not nuclear.

Conclusions – Patients with cryptogenic fibrosing alveolitis have a high frequency of plasma IgG autoantibodies to protein(s) within lung tissue associated with alveolar lining cells. This is believed to be the site where immunological injury

occurs in cryptogenic fibrosing alveolitis, but the significance of these antibodies to the aetiology and pathogenesis is as yet unclear.

(Thorax 1994;49:218–224)

Cryptogenic fibrosing alveolitis is the commonest form of interstitial lung disease and is characterised by progressive pulmonary fibrosis, often leading to death within five years from the time of diagnosis.¹ Histological examination of biopsy material from patients with cryptogenic fibrosing alveolitis has shown a mixed inflammatory cell infiltrate² with an alveolitis affecting the more distal part of the acinar unit.³ Data from bronchoalveolar lavage have shown increased numbers of neutrophils, eosinophils, and CD4 positive lymphocytes,⁴ as well as raised levels of immunoglobulin⁵ in patients with cryptogenic fibrosing alveolitis. Both suggest an immunological pathogenesis for the disease but have not indicated any definite aetiological factors.

In addition there is evidence of a more general immune dysregulation with hypergammaglobulinaemia,⁴ circulating immune complexes,^{6,7} and the production of recognised non-organ specific autoantibodies such as anti-nuclear factor and rheumatoid factor⁸ in some patients. Clinical studies have also shown that the collagen vascular diseases, which have a known immunological pathogenesis involving the production of non-organ specific autoantibodies, may be associated with an identical pattern of alveolitis.⁹

The current consensus is that, whatever the aetiological agent(s), cryptogenic fibrosing alveolitis represents a persistent immunological reaction in the lung with resultant scar formation.¹⁰ The predominance of T lymphocytes in the tissues and in the bronchoalveolar lavage fluid has suggested that the reaction may be mediated by the cellular arm of the immune system. Despite this, markers of T lymphocyte activation have not been found to correlate with disease activity or prognosis.¹¹ It has also been reported that there may be large numbers of B lymphocytes and plasma cells in the lungs of patients with cryptogenic fibrosing alveolitis,¹² the role of which has been largely ignored. While it can be argued that the B lymphocytes are “innocent bystanders” which accompany the T lymphocytes into the tissues, it has been suggested that large numbers of B cells may be associated with a worse prognosis.¹² In addition there is evidence of increased B cell growth factor activity¹³

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and immunoglobulin⁵ in bronchoalveolar lavage fluid from patients with cryptogenic fibrosing alveolitis compared with normal subjects. This indicates possible functional significance for the humoral immune system.

Other better characterised inflammatory disorders of the pulmonary interstitium which may result in scarring, such as sarcoidosis, are also believed to have a T cell mediated pathogenesis. In sarcoidosis there is similar evidence of systemic immune dysregulation, hypergammaglobulinaemia, and accumulation of B lymphocytes and plasma cells within the lung,¹⁴ and it has been shown that such patients have circulating autoantibodies against T lymphocyte epitopes¹⁵ although the significance is unknown.

The presence of autoantibodies in patients with cryptogenic fibrosing alveolitis has been predicted and previously sought^{8,16} but the results have been inconclusive. The most recent study found that 40% of controls and patients with cryptogenic fibrosing alveolitis were positive by an indirect immunofluorescence technique with whole serum on cryostat sections of lung tissue.¹⁶ Other workers using ELISA techniques have found antibodies to the nuclear antigen topoisomerase II (170 kDa) in 38% of patients¹⁷ and hepatitis C virus in 28% of patients.¹⁸

We have readdressed the question of whether cryptogenic fibrosing alveolitis is associated with circulating antibodies to lung proteins by screening the plasma of patients with cryptogenic fibrosing alveolitis, sarcoidosis, and healthy controls using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. In addition we attempted to find the tissue distribution of protein(s) to which patients with cryptogenic fibrosing alveolitis may have autoantibodies.

Methods

PATIENTS

Twenty two newly diagnosed patients with cryptogenic fibrosing alveolitis (14 men, eight women of mean (SE) age 66.2 (1.5) years) were studied. Nineteen of the individuals had undergone open lung biopsy and the remaining three were diagnosed by clinical, radiological, and physiological features. Twenty ml samples of heparinised venous blood were obtained from each of the patients following approval by the local ethics committee. None were on any specific treatment for their disease. The blood sample was centrifuged at 1800 rpm on a Ficoll-Hypaque gradient and the plasma was removed and stored at -70°C .

CONTROLS

Plasma samples were obtained in a similar manner from 17 healthy subjects in the laboratory who had no evidence of respiratory symptoms and who were on no medication (10 men and seven women of mean (SE) age 41.5 (2.5) years), and 22 patients with histologically confirmed sarcoidosis (13 men and nine women of mean (SE) age 43.4 (2.2) years).

PREPARATION OF PROTEIN EXTRACTS

Protein extracts were prepared from open lung biopsy material obtained from eight patients with cryptogenic fibrosing alveolitis and three with sarcoidosis. Control tissues were obtained from histologically normal lung resection tissue, colonic mucosa, skin, liver, and tonsil.

The tissue was collected fresh in theatre, snap frozen in 1 ml 1% Nonidet P-40 lysis buffer (a non-ionic detergent), and stored at -70°C . The specimen, still in lysis buffer, was then mechanically disrupted while still frozen with a mortar and pestle, and transferred to a universal container. A further 1 ml aliquot of lysis buffer was then added and the container placed in an ultrasonic water bath for three minutes. The sample was then placed in an ultracentrifuge, spun at 32 000 rpm for 10 minutes, and the protein containing supernatant removed and kept on ice.

The protein content of the supernatant was assayed using a colorimetric technique (BioRad Labs Ltd, UK) and diluted 1:2 with sample buffer (3.6 ml distilled water, 1.0 ml 0.5 mol/l Tris-HCl, pH 6.8, 10% w/v SDS, 0.8 ml 1 mol/l dithiothreitol, 0.8 ml glycerol, 0.05 ml 0.05% w/v bromophenol blue) and stored at -70°C .

SDS-PAGE ELECTROPHORESIS

The Mini-Protein II polyacrylamide gel electrophoresis system (BioRad Labs Ltd, UK) was used throughout and the manufacturer's protocol followed. 7.5% acrylamide running gels with 4% acrylamide preparative (with one reference well) stacking gels were produced according to the manual. The gels were loaded with 2 mg of a single protein extract alongside markers of known molecular weight in the reference well. The sample was run under reducing conditions using a constant 200 volt supply until the bromophenol blue band was seen to reach the bottom of the gel. The proteins were then electrophoretically transferred to nitrocellulose paper (Hybond C, Amersham, UK) and stored at 4°C . The gels were stained with 0.2% w/v Coomassie blue to ensure that protein separation had occurred.

WESTERN BLOTTING

Before blotting the nitrocellulose sheets were blocked for one hour at room temperature (5% w/v dried milk powder in Tris buffered saline (TBS)) followed by three five minute washes in Tris buffered saline with 0.1% Tween 20 (TTBS). The test sera were diluted 1:50 with antibody buffer (1% w/v dried milk powder in TTBS). The nitrocellulose sheets were then incubated for 30–60 minutes with the patient and control plasma at room temperature in a multichannel developing clamp (BioRad Labs Ltd, UK) which divided the membrane into distinct channels allowing up to 20 different sera to be tested simultaneously against one protein extract. The monoclonal antibodies against the leucocyte common antigen, CD45, or vimentin (both Dako UK Ltd), at 1:500

Summary of Western blotting data for the experiments presented indicating the frequency of plasma samples from each test group which contain autoantibodies recognising a 70–90 kDa antigenic protein derived from the different tissue extracts

Tissue source	Frequency of plasma samples recognising a 70–90 kDa protein in tissue extracts		
	CFA plasma	Sarcoid plasma	Control plasma
CFA lung biopsy	18/22 (82%)*	5/18 (28%)	1/17 (6%)
Sarcoid lung biopsy	18/19 (95%)*	6/22 (27%)	5/17 (23%)
Control lung	12/15 (80%)	ND	ND
Colonic mucosa	3/13 (23%)†	ND	ND
Liver	3/14 (21%)†	ND	ND
Tonsil	3/14 (21%)†	ND	ND
Skin	4/14 (29%)†	ND	ND

CFA = cryptogenic fibrosing alveolitis; ND = not done.

* Frequency of positive bands in the 70–90 kDa range is significantly more frequent ($p < 0.001$) with the plasma samples from patients with cryptogenic fibrosing alveolitis than the sarcoid or control plasma samples.

† Frequency of positive bands in the 70–90 kDa range is significantly less frequent than on the control lung extract ($p < 0.05$) or the interstitial lung disease extracts ($p < 0.001$).

dilution were used as positive controls and antibody buffer alone as a negative.

The membranes were removed from the clamp, given three five minute washes in TTBS, and then developed for adherent antibodies by incubation with murine antihuman IgG_γ (Sigma Chemical Co, UK, clone no. GG5 at 1:1000), antihuman IgM_μ (clone no. DA6 127, kindly donated by Dr K Guy), or antihuman IgA_γ (Sigma Chemical Co, UK, clone no. GA-112). The membranes were washed as above, incubated with biotinylated rabbit anti-mouse antibody (Dako UK Ltd), diluted 1:1000 for 30 minutes, and washed again. This was followed by a further 30 minute incubation with an alkaline phosphatase conjugated avidin-biotin complex (Dako UK Ltd) diluted 1:30 and further washing. Visualisation was carried out using the NBT/BCIP substrate (Sigma Chemical Co, UK) in an alkaline phosphatase buffer. The membranes were then blotted dry with tissue paper and stored.

The molecular weights for the positive bands in each sample were calculated from a standard curve produced from the molecular weight markers.

PURIFICATION OF IgG FROM PLASMA

The IgG fraction in plasma samples from six patients and two controls was purified from the whole sample using protein G (Pharmacia) following the manufacturer's protocol. The concentration of the purified IgG was measured as described above, aliquoted, and stored at -70°C .

STAINING OF TISSUE SECTIONS WITH PURIFIED IgG

Cryostat sections of lung from patients with cryptogenic fibrosing alveolitis obtained at open lung biopsy were cut, fixed with acetone, and washed with TBS. The sections were blocked with protein A (Sigma Chemical Co, UK) for 30 minutes, washed with TBS, and then further blocked with fresh normal human serum for one hour. They were then incubated with 20 μg of the purified IgG from either a patient with cryptogenic fibrosing alveolitis or healthy control for one hour at room temperature, followed by further washing with TBS. A final incubation was then performed with a protein A-alkaline phosphatase conjugate (Sigma Chemical Co, UK). Visualisation was performed using Vector Red (Vector Lab, UK) with levamisole to block endogenous alkaline phosphatase activity. This substrate is visible by both light and ultraviolet microscopy. The sections were counterstained, mounted, and viewed with a confocal laser microscope (Zeiss) in ultraviolet mode with filters to reduce autofluorescence from collagen and elastin.

STATISTICS

All statistical analyses were performed with the χ^2 test.

Results

The results obtained for IgG autoantibodies are summarised in the table. No evidence of reactive IgM or IgA autoantibodies was found.

CRYPTOGENIC FIBROSING ALVEOLITIS PROTEIN EXTRACT

Cryptogenic fibrosing alveolitis (fig 1) and control (fig 2) plasma samples were blotted against protein derived from cryptogenic fibrosing alveolitis biopsy material to identify any evidence of autoantibodies that were specific for cryptogenic fibrosing alveolitis. The results showed bands extending across the blot as the result of non-specific reactivity of the detection system with immunoglobulin and biotin present in the original lung tissue. In addition many individuals showed antibodies reacting with several proteins of differing molecular weights. These represent naturally

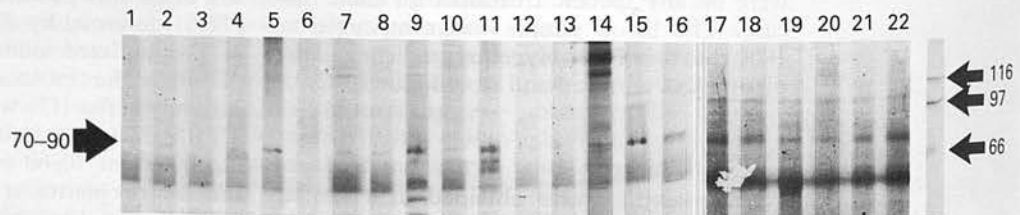


Figure 1 Illustration of Western blot results obtained by blotting the 22 plasma samples from patients with cryptogenic fibrosing alveolitis on a protein extract produced from cryptogenic fibrosing alveolitis lung biopsy material. The molecular weight markers are indicated in kDa on the right. Positive bands of varying intensity are identified in the 70–90 kDa range for 18 of the 22 plasma samples (lanes 2, 4, 5, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18, 19, 20, 21, and 22). The biopsy material in the blot shown corresponds to the plasma shown in lane 22. Other bands visible on the blot are the result of non-specific reactions between the detection system and immunoglobulin or biotin in the tissue extract, or the presence of naturally occurring autoantibodies which are also found in the controls and as such have no pathological significance.

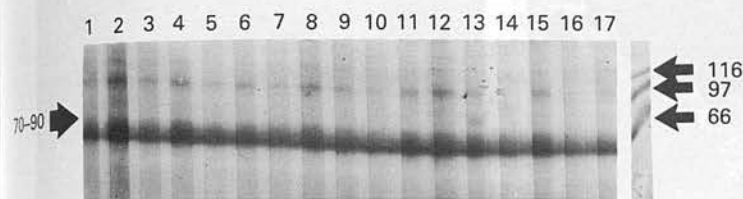


Figure 2 Illustration of a Western blot showing the results obtained blotting the 17 control plasma samples on a protein extract produced from cryptogenic fibrosing alveolitis lung biopsy material. The molecular weight markers are indicated in kDa on the right. Only one sample (lane 13) shows positivity in the 70–90 kDa range as indicated on the left.

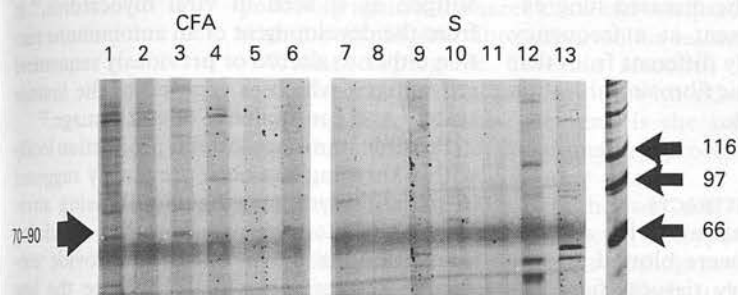


Figure 3 Illustration of a Western blot showing representative results obtained blotting cryptogenic fibrosing alveolitis (lanes 1–6) and sarcoid (lanes 7–13) plasma samples on a protein extract produced from sarcoid lung tissue. The molecular weight markers are indicated in kDa on the right. Five of the six cryptogenic fibrosing alveolitis plasma samples illustrated are positive in the 70–90 kDa range (lanes 1, 2, 3, 4, and 6) compared with only one of the seven sarcoid plasma samples (lane 10).

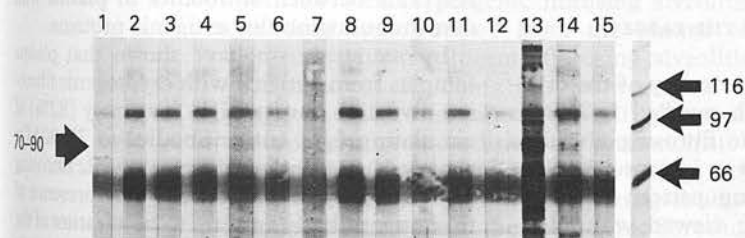


Figure 4 Illustration of a Western blot showing the results obtained blotting 15 cryptogenic fibrosing alveolitis plasma samples on a protein extract produced from control, non-interstitial lung disease, lung tissue. The molecular weight markers are indicated in kDa on the right. The positive bands are very much fainter than in fig 1 but are present in 12 of the 15 plasma samples in the 70–90 kDa range (lanes 1, 2, 3, 4, 5, 7, 8, 10, 11, 13, 14, and 15).

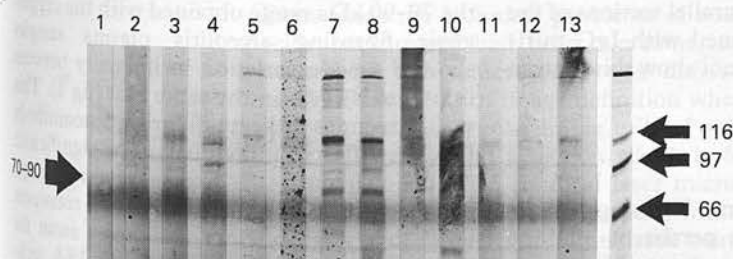


Figure 5 Illustration of a Western blot showing the results obtained blotting 13 cryptogenic fibrosing alveolitis plasma samples on a protein extract produced from colonic mucosa. The molecular weight markers are indicated in kDa on the right. Positive bands are identified in the 70–90 kDa range in three of the 13 plasma samples (lanes 7, 8, and 13).

occurring autoantibodies with no pathological significance as they are seen in both patient and control groups. Only bands in the 70–90 kDa range were found to have a significant correlation with the cryptogenic fibrosing alveolitis samples (cryptogenic fibrosing alveolitis plasma samples 18 of 22; control plasma samples one of 17, $p < 0.001$). Similar results were obtained using eight different cryptogenic fibrosing alveolitis biopsy samples as a source of protein extract.

To determine whether autoantibodies to these protein(s) were specific to the plasma samples from patients with cryptogenic fibrosing alveolitis or represented a non-specific response to inflammation in the pulmonary interstitium, 18 plasma samples from patients with sarcoidosis were blotted against the same extract. Only five of these 18 samples contained autoantibodies which recognised protein(s) in the 70–90 kDa range. This was significantly fewer than for the cryptogenic fibrosing alveolitis plasma samples ($p < 0.001$), but not significantly different from the controls.

SARCOID PROTEIN EXTRACT

As we had found evidence that cryptogenic fibrosing alveolitis plasma samples contained a high frequency of autoantibodies to 70–90 kDa lung protein(s) present in lung tissue from patients with cryptogenic fibrosing alveolitis, it was of interest to determine whether the same protein(s) were present in lung tissue from other sources. We chose to use extracts from lung biopsies taken from patients with sarcoidosis as this represented another inflammatory condition affecting the pulmonary interstitium with a different pattern of disease and prognosis.

The same plasma samples were blotted onto protein extracts derived from biopsy material from patients with sarcoidosis (fig 3). Eighteen of 19 cryptogenic fibrosing alveolitis plasma samples were shown to react with 70–90 kDa protein(s) compared with only six of 22 sarcoid plasma samples ($p < 0.001$). The frequency of positivity to 70–90 kDa protein(s) was not significantly different from that obtained using the cryptogenic fibrosing alveolitis extract for either group. Five of 17 control plasma samples were positive on the 70–90 kDa region on the sarcoid extract, and this was found to be significantly more frequent than on the cryptogenic fibrosing alveolitis extract ($p < 0.05$).

CONTROL LUNG EXTRACT

To determine whether the protein(s) detected by the autoantibodies in the plasma from patients with cryptogenic fibrosing alveolitis were normal lung constituents or only present in patients with inflammatory lung disease, cryptogenic fibrosing alveolitis plasma samples were blotted against protein extracts from control lung tissue. Twelve of the 15 cryptogenic fibrosing alveolitis plasma samples were positive, recognising 70–90 kDa protein(s) in the extract (fig 4). The bands obtained were very

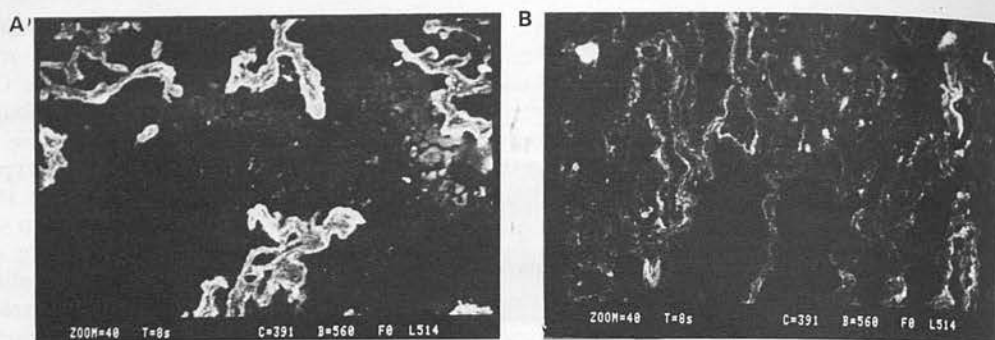


Figure 6 Photomicrograph obtained using the confocal laser microscope in ultraviolet mode of (A) a cryostat section of cryptogenic fibrosing alveolitis lung biopsy material stained with purified IgG (20 µg) from a cryptogenic fibrosing alveolitis plasma sample. The linear staining along the edge of the alveoli can be seen in a distribution that suggests the antigenic proteins detected by the autoantibodies are associated with alveolar lining cells in the lung parenchyma; and (B) a cryostat section of cryptogenic fibrosing alveolitis lung biopsy material stained with purified IgG (20 µg) from a control plasma sample. Background autofluorescence from the collagen and elastin structures in the lung parenchyma can be seen but the linear staining observed in (A) is absent.

much fainter than with the diseased lung extracts but were still present at a frequency which was not significantly different from that obtained on the cryptogenic fibrosing alveolitis or sarcoid protein extracts.

NON-PULMONARY TISSUE EXTRACTS

Plasma samples from patients with cryptogenic fibrosing alveolitis were blotted against extracts of non-pulmonary tissues (fig 5) to ascertain if the protein(s) detected were lung specific. The frequency of positivity obtained (normal colonic mucosa three of 13; liver three of 14; skin four of 14) was significantly lower than on the control lung extract ($p < 0.05$) and lung extracts from patients with interstitial lung disease ($p < 0.001$).

IMMUNOLocalISATION OF THE TARGET PROTEIN(S)

Examination by light microscopy of the cryostat sections stained with purified IgG from patients with cryptogenic fibrosing alveolitis and controls was difficult to interpret because of the fine linear staining pattern obtained. When the sections were viewed with ultraviolet light using a confocal laser microscope the IgG purified from the plasma of patients with cryptogenic fibrosing alveolitis was observed to show a fine linear staining pattern along the surface of alveolar septa (fig 6A). The distribution suggested that the target protein(s) detected may be associated with alveolar lining cells. The staining pattern was not nuclear but cytoplasmic or membranous, or both, in its distribution. Parallel sections of the same biopsy material stained with IgG purified from the controls did not show this pattern of positivity (fig 6B).

Discussion

In patients with cryptogenic fibrosing alveolitis there is evidence of a persistent immune reaction in the pulmonary interstitium with resulting fibrosis.² The mechanisms which drive these chronic responses are unknown but theoretically could result from either persistence of an extrinsic triggering (possibly viral)

antigen as is seen in viral myocarditis,¹⁹ or from the development of an autoimmune reaction either to altered or previously sequestered self-antigen which is exposed to the immune system as a consequence of cell damage.²⁰

Possible immunoglobulin production locally within the lung has been previously suggested in patients with cryptogenic fibrosing alveolitis²¹ but attempts to identify circulating autoantibodies have failed to provide conclusive results, principally because the lung contains large amounts of cell bound immunoglobulin to which labelled antihuman immunoglobulin sticks when applied to cryostat sections. The technique of Western blotting circumvents this problem as the proteins present in the lung, including immunoglobulins, are separated according to molecular weight. This allows identification of specific reactions between antibodies in plasma with non-immunoglobulin antigenic proteins.

In our study we have shown that plasma samples from patients with cryptogenic fibrosing alveolitis have a high frequency (82%) of circulating IgG autoantibodies to 70–90 kDa protein(s) present within cryptogenic fibrosing alveolitis biopsy lung tissue. The presence of this autoantibody appears to be a feature of the plasma samples from patients with cryptogenic fibrosing alveolitis, in comparison with the sarcoid and control plasma samples. The 70–90 kDa protein(s) within the tissue extracts, however, were not specific to the cryptogenic fibrosing alveolitis extract and could be seen also in extracts of sarcoid and control lung tissue.

As might be expected, the positive bands in the 70–90 kDa range obtained with the cryptogenic fibrosing alveolitis plasma samples showed a wide variation in intensity between individuals even on the same blot (fig 1). This would seem to indicate differing autoantibody titres in different individuals. The significance of this in terms of disease severity, stage, prognosis, and possible response to treatment is at this stage unclear and must await the development of a quantitative ELISA technique.

The control plasma samples recognised 70–90 kDa protein(s) significantly more frequently in the sarcoid protein extract than the

cryptogenic fibrosing alveolitis extract. The explanation for this is unclear, but may relate to the relative amounts of lung epithelial tissue present in the two biopsy types. In biopsy material from patients with cryptogenic fibrosing alveolitis a larger proportion of the tissue volume is likely to be occupied by scar tissue than in biopsy material from patients with sarcoidosis; thus the relative contribution of lung epithelial proteins may be greater in the sarcoid extract than the cryptogenic fibrosing alveolitis extract. Since most autoantibodies occur at low titre in normal individuals, this possible relative increase in the amount of epithelial cell derived protein in the extract may explain the small rise in background frequency observed against sarcoid extracts. Interestingly, the sarcoid plasma samples did not show such an increase in frequency of positivity against the sarcoid extract compared with the cryptogenic fibrosing alveolitis extract.

Whilst cryptogenic fibrosing alveolitis is recognised as having associations with other diseases, in most cases the lung is the sole organ affected by the inflammatory process, unlike sarcoidosis which may present as a multiorgan disease. We hypothesised that if the autoantibodies we have detected in cryptogenic fibrosing alveolitis have a pathogenetic role, then the target protein(s) are likely to be lung specific. We have shown that most patients with cryptogenic fibrosing alveolitis who have autoantibodies recognising 70–90 kDa protein(s) in lung tissue fail to recognise protein(s) of similar weight in four non-pulmonary tissues.

In order to identify possible site(s) of the antigenic protein(s) in biopsy material from patients with cryptogenic fibrosing alveolitis we used IgG purified from the plasma of patients with cryptogenic fibrosing alveolitis and controls. By doing this we were able to apply a standard amount of IgG (20 µg) to each section, thus standardising the immunohistochemical technique and circumventing any non-specific effect of hypergammaglobulinaemia on the results in the cryptogenic fibrosing alveolitis patients. The tissue staining with purified IgG was obtained by using a protein A-alkaline phosphatase conjugate which binds IgG specifically by the Fc portion of the molecule as a second layer. This technique allowed the omission of an antihuman immunoglobulin and thus removed one of the technical problems that has prevented such studies being successful in the past.

Even when the technical problems of non-specific background had been overcome, assessment by light microscopy was difficult because of problems of image definition when examining stained alveolar lining cells of only 2–3 µm in diameter on a section which is 6–8 µm in thickness. The confocal laser microscope is a high definition instrument which views an optical section within the tissue being examined. This resulted in removal of many of the optical limitations presented by the sections and optimised the image obtained. The fine, linear pattern of staining observed with the cryptogenic fibrosing alveolitis derived

IgG (but not the control IgG) strongly suggested that the protein(s) recognised by the autoantibodies were in association with alveolar lining cells. Previous studies have suggested that this is the site of immunological damage in cryptogenic fibrosing alveolitis²² and immune complexes have been reported on the alveolar surface.^{23,24}

We hypothesised that the autoantibodies we had detected in patients with cryptogenic fibrosing alveolitis could be directed against either self-antigen or exogenous antigen expressed in the lungs of patients with cryptogenic fibrosing alveolitis. The data we have presented here show that the protein(s) detected by the cryptogenic fibrosing alveolitis autoantibody are present in both patients with sarcoidosis and control non-interstitial lung disease tissue, suggesting that they are endogenous rather than exogenous. The staining pattern obtained on the control lung tissue extracts was weaker than on the interstitial lung disease lung extracts. This could be due to increased cellular expression as a result of the disease process, or an increase in the number of cells in the tissue expressing it. Recent studies of rat type I and II lung epithelial cells in culture²⁵ have shown the presence of a variably glycosylated cell surface protein of 74–88 kDa which is present on the surface of both cell types. Inflammatory conditions of the lung, particularly cryptogenic fibrosing alveolitis, are known to be associated with proliferation and increased numbers of type II pneumocytes and this may be relevant to our findings.

We have also considered the possibility that the autoantibodies are detecting heat shock proteins of which there are many in this weight range.²⁶ Patients with sarcoidosis are, however, reported as having a high frequency of detectable antibodies to heat shock proteins,²⁷ but in our study plasma samples from patients with sarcoidosis did not show significant reactivity to the 70–90 kDa protein(s). Nor do we believe that the antigen is directed against immunologically active molecules such as MHC class II, which may be inducible on epithelial cells at sites of inflammation or immunoglobulin, as the frequency of positivity of plasma from patients with cryptogenic fibrosing alveolitis on tonsillar extracts, which contains an abundance of such molecules, was significantly lower than on the control lung tissue.

Our results have shown that patients with cryptogenic fibrosing alveolitis have a high incidence of circulating IgG autoantibodies to endogenous lung protein(s) which appear to be associated with alveolar lining cells. Most of these cases fail to recognise protein(s) of similar weight in non-pulmonary tissues, suggesting that it may be relatively lung-specific in its distribution. The antigenic protein(s) would appear to be upregulated in both lung tissue from patients with cryptogenic fibrosing alveolitis and sarcoidosis, although the latter group do not seem to mount a humoral immune response. This suggests that the development of these autoantibodies is not simply the result of upregulation or release of

sequestered protein(s) due to inflammation in the lung, but that other factors – possibly reflecting differences in the immunological pathogenesis of the two diseases – are involved.

We believe that this study represents the first conclusive evidence of circulating antibodies to protein(s) present in the lung in patients with cryptogenic fibrosing alveolitis. The significance of this finding with regard to our understanding of the aetiology and pathogenesis of cryptogenic fibrosing alveolitis is, at this stage, unclear, but it reinforces the view that the role of the humoral immune system in the pathogenesis of cryptogenic fibrosing alveolitis deserves further consideration.

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Localisation of a pulmonary autoantigen in cryptogenic fibrosing alveolitis

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Abstract

Background - Cryptogenic fibrosing alveolitis (CFA) is believed to have an immunological pathogenesis with a persisting inflammatory reaction to an as yet unidentified pulmonary antigen(s). A high frequency of IgG autoantibodies has previously been found in the plasma of patients with CFA to an extractable 70-90 kDa lung antigen by Western blotting. Preliminary immunohistochemical studies with patient IgG had indicated that the target protein(s) might be associated with alveolar epithelial lining cells which have previously been suggested as the site of immunological attack in CFA.

Methods - In order to confirm this finding immunohistochemical analysis and Western blotting were performed on a human type II alveolar cell line (A549) using CFA patient plasma. In order to study further the distribution of the antigen, antibodies were raised in a rabbit to the partially purified 70-90 kDa CFA lung protein.

Results - The results showed that the human CFA autoantibody recognised a 70-90 kDa protein with a cytoplasmic distribution present in the A549 cells, confirming previous observations. The immune rabbit IgG recognised a protein of similar molecular weight by Western blotting of protein derived from lung biopsy samples of patients with CFA and A549 cells. In addition it immunoprecipitated protein(s) of this molecular weight from lung biopsy protein extracts from patients with CFA. The precipitated protein(s) were found to cross-react with the autoantibody found in the plasma of patients with CFA. Immunohistochemical analysis with the immunised rabbit antibody revealed positive staining of type I and II alveolar epithelial lining cells in CFA. A similar pattern of epithelial staining was also observed with the rabbit IgG on biopsy specimens of lung from patients with sarcoidosis and control lung tissue, although this was more focal and less intense. No positive staining was seen on sections from a number of non-pulmonary tissues (colon, liver, kidney, tonsil, lymph node, skin, cervix). Cytoplasmic staining of the A549 cell line was also detected.

Conclusions - The 70-90 kDa protein recognised by autoantibodies in patients with CFA is associated with pulmonary epithelial lining cells. The immune rabbit IgG produced appears to recognise antigen

by Western blotting and immunohistochemical staining of lung tissue in a similar pattern to the patient autoantibodies. Immunohistochemical data obtained with this antibody suggest that the putative autoantigen against which patients with CFA mount a humoral immune response may be endogenous and specific to the lung.

(Thorax 1994;49:1139-1145)

Cryptogenic fibrosing alveolitis (CFA) is characterised by progressive pulmonary fibrosis¹ often leading to death within five years from the time of diagnosis.² Although the aetiology remains uncertain, CFA is believed to have an immunologically mediated pathogenesis with a persisting inflammatory reaction to as yet unidentified antigen(s) within the lung.³ Research interest has focused principally on the role of the cellular arm of the immune system and the interaction of T lymphocytes, monocytes, and lung fibroblasts in this disease process.⁴

We have recently described the presence of circulating IgG autoantibodies in patients with CFA which recognise a 70-90 kDa lung antigen on Western blot analysis.⁵ These studies indicated that the antigen was likely to be endogenous as it was detectable in lung tissue from patients with sarcoidosis and in those with no evidence of interstitial lung disease. Preliminary immunohistochemical studies using IgG purified from patient and control plasma samples suggested that the antigen was associated with the alveolar lining cells of the lung. Whilst the role of these antibodies in the pathogenesis of the disease is unclear, morphological studies have suggested that alveolar lining cells may be the principal target of immunological attack in CFA⁶ and previous reports have detected immune complexes at this site.⁷

In this study we report the results of experiments performed using an immunised rabbit IgG raised against the partially purified human 70-90 kDa lung protein. These experiments were designed to confirm our initial observations that the antigenic protein(s) detected by patient autoantibodies are associated with alveolar epithelial lining cells by studying tissue sections and using a human alveolar epithelial cell line (A549).⁸

Methods

CFA PATIENT AND CONTROL PLASMA SAMPLES
Twenty ml heparinised venous blood was obtained from 17 patients with histologically con-

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firmed CFA (11M:6F, median age (range) 68 (49–80) years) following approval by the local medical ethics committee. All the samples were obtained at the time of biopsy and none of the patients were on medication specific for their disease. The plasma fraction from each sample was recovered, aliquoted, and stored at -70°C .

Plasma samples were obtained in an identical fashion from 17 healthy volunteers within the Department of Pathology who had no evidence of respiratory symptoms and were on no medication (10M:7F, median age (range) 42 (28–58) years).

PREPARATION OF CFA LUNG BIOPSY PROTEIN EXTRACTS

Protein extracts were produced from tissue obtained at open lung biopsy from patients with histologically confirmed CFA by a method we have previously described.⁵ The tissue was obtained fresh and snap frozen in lysis buffer containing 1% Nonidet P-40. It was then mechanically disrupted using a mortar and pestle and transferred to a universal container. A further 1 ml aliquot of lysis buffer was added and the container placed in an ultrasonic water bath for three minutes. The sample was then ultracentrifuged at 32 000 rpm for 10 minutes and the protein-containing supernatant removed and kept on ice. The protein content was then estimated using a commercially available colorimetric assay (BioRad UK).

A549 CELL LINE: PREPARATION OF PROTEIN EXTRACTS AND CYTOSPINS

The human A549 type II alveolar cell line⁸ was grown in RPMI medium (Gibco BRL Ltd, UK) supplemented with 10% heat inactivated fetal calf serum (Gibco BRL Ltd, UK) in 5% CO_2 at 37°C . When confluent the monolayers were washed with phosphate buffered saline and harvested using a non-enzymatic cell dissociation buffer (Gibco BRL Ltd, UK). The harvested cells were then pooled and protein extracts produced for immunohistochemical analysis by making cytospin preparations at a density of 5×10^4 per slide followed by acetone fixation and storage at -20°C .

POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOTTING

The methods used in these experiments have been described in detail elsewhere.⁵ Briefly, protein extracts produced from CFA open lung biopsy specimens and A549 cell pellets were run on 7.5% acrylamide gels under reducing conditions according to the manufacturer's protocol (BioRad Ltd, UK). The separated proteins were transferred to nitrocellulose sheets (Amersham, UK) and blotted with either human plasma or rabbit IgG.

Interaction between antibodies in human plasma and protein on the nitrocellulose was detected by an avidin-biotin alkaline phosphatase detection system which we have described elsewhere.⁵ Interaction between rabbit IgG and the protein was carried

out by a similar method using a biotin conjugated swine anti-rabbit antibody (1:500; Dako UK Ltd) followed by the avidin-biotin-alkaline phosphatase complex (Dako UK Ltd). Visualisation in both cases was performed using the NBT/BCIP substrate (Sigma UK).

PRODUCTION OF ANTI-HUMAN SERUM

Partially purified antigen was obtained from lung biopsy material from patients with CFA for immunisation in two separate ways as we were unsure how antigenic the material produced would be.

(a) Protein extracts pooled from open lung biopsies from patients with CFA were separated by molecular weight on 7.5% polyacrylamide gels and transferred to nitrocellulose sheets as described above. The position of the 70–90 kDa protein(s) membrane on the membrane was identified by blotting one edge. The strip containing the protein(s) was then excised, solubilised in dimethylsulphoxide at 37°C , and reprecipitated with phosphate buffered saline (PBS) to give a fine particulate suspension of protein(s) bound to nitrocellulose.⁹

(b) IgG was purified from patient plasma using protein G (Pharmacia) in a manner we have previously described.⁵ This was then used to construct an affinity purification column by linking the IgG to cyanogen bromide activated Sepharose beads (Pharmacia Ltd, UK) according to the manufacturer's protocol. The beads were then incubated at 4°C with protein extracts derived from CFA open lung biopsy samples overnight on a roller mixer. This technique resulted in the production of particulate Sepharose-IgG-protein(s) complexes which were recovered by centrifugation and washed with PBS.

The protein-nitrocellulose and Sepharose-IgG-protein complexes were combined and mixed with an equal volume of alum adjuvant (Pierce and Warriner) for immunisation. One female Dutch White rabbit (obtained from Hy-line Commercial Rabbits Ltd, UK) was immunised by administering 1.5 ml of the mixture subcutaneously at two sites following the collection of baseline serum samples to act as non-immune controls. A total of three such injections were given at six weekly intervals following which test serum samples were obtained.

The control (baseline) and immunised serum samples were absorbed against human IgG-coated agarose beads to remove any anti-human IgG reactivity. IgG was then purified from both samples using a Sepharose-protein A column (Pharmacia Ltd, UK) and adjusted to give a final total IgG concentration of 1 mg/ml.

IMMUNOPRECIPITATION

Immunoprecipitation of the antigenic protein from lung tissue obtained from patients with CFA was performed using the immunised rabbit serum according to an established protocol.¹⁰ One hundred and fifty μl (2.25 mg) of

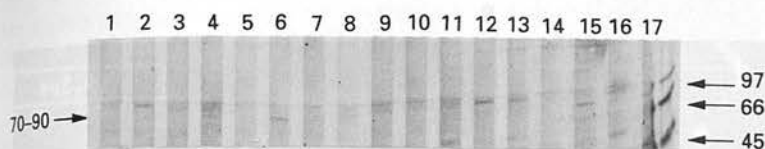


Figure 1 Western blot showing the results obtained by blotting 17 plasma samples obtained from patients with CFA on a protein extract produced from the A549 human type II alveolar epithelial cell line. The molecular weight markers are indicated in kDa on the right. Positive bands are identified in the 70–90 kDa range in 12 of the 17 plasma samples (lanes 1, 2, 3, 4, 6, 8, 11, 12, 13, 15, 16, and 17).

pooled protein extract, derived from four CFA open lung biopsy samples as described above, was incubated on ice with 7.5 μ l control rabbit serum to preclear the extract. The rabbit immunoglobulin and any complexes that had formed were removed by adding 100 μ l of a 10% w/v suspension of protein A-conjugated Sepharose beads and incubating on a rocker at room temperature for 30 minutes. The mixture was then ultracentrifuged at 13 000 rpm for 10 minutes, the supernatant retained, and the beads discarded. Immunised rabbit serum was added to give a 1:10 dilution of serum in the protein extract solution. This was incubated for two hours on ice. A further 100 μ l of the 10% protein A-Sepharose suspension was added to recover the immunised rabbit immunoglobulin antigen complexes. The mixture was incubated for two hours at room temperature on a rocker and the beads with attached immunoglobulin and complexes were recovered by ultracentrifugation at 13 000 rpm for 10 minutes. The beads were then washed twice in high salt buffer, twice in low salt buffer, and once in a no salt buffer prior to boiling for five minutes in a polyacrylamide gel electrophoresis sample buffer (3.6 ml distilled water; 1.0 ml 0.5 mol/l Tris-HCl pH 6.8; 10% w/v SDS; 0.8 ml 1 mol/l dithiothreitol; 0.8 ml glycerol; 0.05 ml 0.05% w/v bromophenol blue) to release the immunoglobulin from the beads and break the complexes with the precipitated cellular proteins. The sample was centrifuged at 13 000 rpm for 10 minutes, the supernatant removed and run on 7.5% polyacrylamide gels under reducing conditions. The proteins were transferred to nitrocellulose membranes as described above. The polyacrylamide gels were then stained with Coomassie Blue (Sigma UK) to determine whether any protein had been precipitated.

To determine if antigen precipitated from the lung biopsy extracts with the rabbit serum samples was recognised by the human auto-antibody the membrane was blotted with patient plasma. Since the detection system employed utilised a monoclonal anti-human IgG γ which we knew to crossreact with rabbit IgG (WAH Wallace, personal observation), a second parallel blot was performed omitting the patient plasma. This step was required to identify the position of the rabbit IgG on the blot and to differentiate precipitated human pulmonary proteins from rabbit immunoglobulin.

IMMUNOHISTOCHEMISTRY WITH IgG FROM PATIENTS WITH CFA AND CONTROLS

Acetone-fixed cytospin preparations of A549 cells were stained with purified IgG from serum

samples from patients with CFA and healthy controls using a technique we have previously described.⁵ Briefly, the specimens were incubated with the IgG at a standard concentration (0.02 μ g/100 μ l) and adherent antibody was detected using a protein A-alkaline phosphatase conjugate (Sigma UK) followed by visualisation with Vector Red (Vector Laboratories UK). Sections were then viewed on a confocal laser microscope (Zeiss).

IMMUNOHISTOCHEMISTRY WITH RABBIT IgG

Acetone-fixed cryostat sections were obtained from eight cases of CFA, two cases of sarcoidosis, and from two specimens from pneumonectomy procedures performed for malignant disease and showing no histological evidence of interstitial lung disease. Sections were also obtained and processed in an identical manner from histologically normal skin, tonsil, lymph node, colonic mucosa, cervix, liver, and kidney. Cytospin preparations of A549 cells, peripheral blood mononuclear cells, human T cell lines (H9 and Jurkat), and primary cultured chondrocytes were obtained in a similar fashion from other groups in the laboratory and prepared as described above.

All the cases were stained with the control and immunised rabbit IgG diluted 1:10 using a standard ABC-alkaline phosphatase method. Visualisation was performed using the Vector Red substrate with the inclusion of 1 mmol/l levamisole to block endogenous alkaline phosphatase activity. For each case a further section was included where the rabbit IgG was omitted to act as a negative control. The sections were counterstained, dehydrated, mounted, and viewed by light microscopy.

IMMUNOHISTOCHEMICAL DEMONSTRATION OF IMMUNE COMPLEXES IN LUNG TISSUE FROM PATIENTS WITH CFA

Identification of immune complexes in lung biopsy specimens obtained from patients with CFA was carried out using an antibody to C1q, an activated fragment of complement. Formalin-fixed paraffin embedded biopsy tissue was obtained from the departmental archives from 12 cases of CFA. Sections from these cases were stained using a standard indirect immunoperoxidase method with an antibody to C1q (1:500, Dako UK) and visualisation with diaminobenzidine (Sigma UK). Endogenous peroxidase activity was blocked with H_2O_2 .

Results

DETECTION OF ANTIGEN IN A549 CELLS BY WESTERN BLOTTING AND IMMUNOHISTOCHEMISTRY WITH PLASMA OBTAINED FROM PATIENTS WITH CFA

Figure 1 shows the results obtained from blotting plasma samples from 17 patients against protein derived from the A549 cell line. Of the 17 patients 12 (71%) had IgG which recognised a protein in the 70–90 kDa range on the blot compared with only three (18%) of the 17 healthy control plasma samples ($p < 0.01$, χ^2

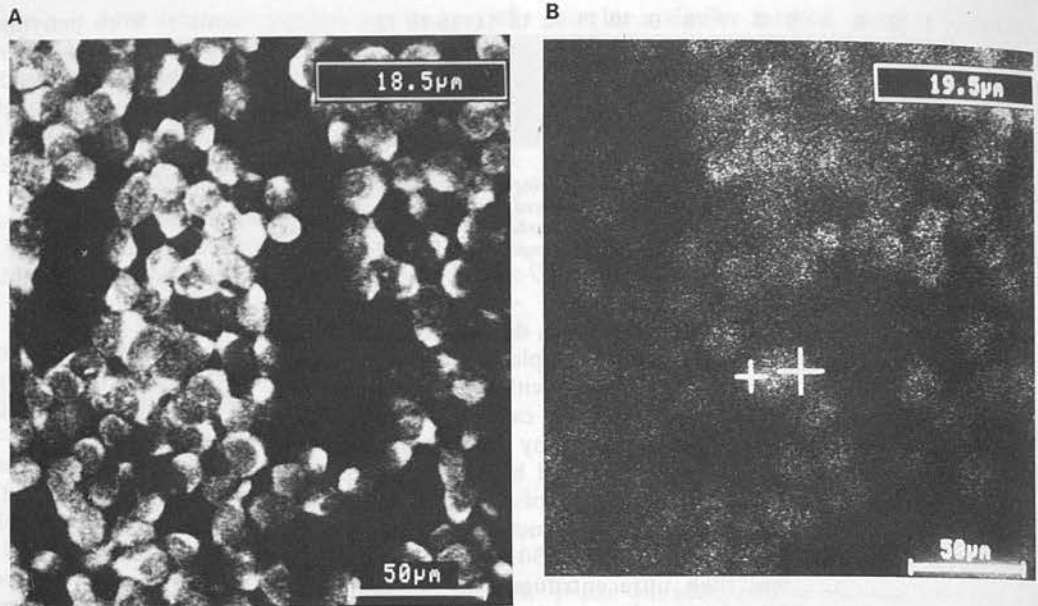


Figure 2 Photomicrograph obtained using the Confocal laser microscope in ultraviolet mode of A549 cells stained with purified IgG (20 μ g) from (A) plasma obtained from patients with CFA and (B) control plasma. Strong cytoplasmic staining of the cells can be seen with the CFA IgG which is not present in the slide synchronously stained with the control IgG.

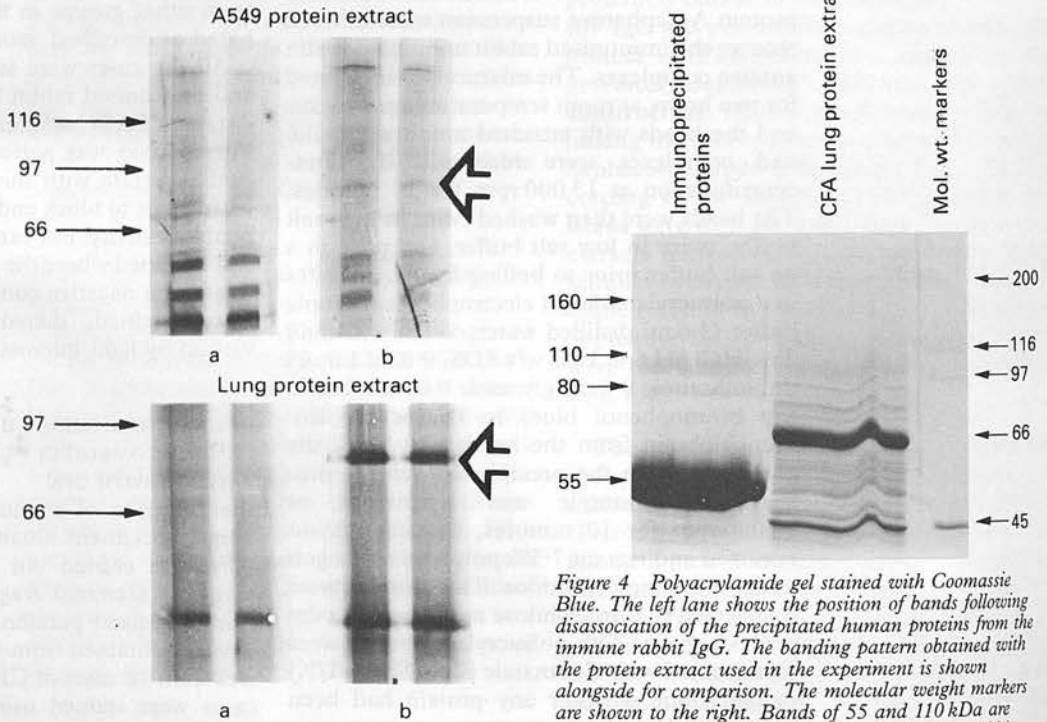


Figure 3 Western blots obtained with the non-immunised control rabbit IgG (a) and the immunised rabbit IgG (b) with protein extracts obtained from A549 cell pellets and lung biopsy tissue obtained from patients with CFA. The molecular weight markers are shown on the left. Positive bands can be seen on both blots with the immune IgG in the 70–90 kDa range (open arrows) that are absent with the control IgG.

Figure 4 Polyacrylamide gel stained with Coomassie Blue. The left lane shows the position of bands following disassociation of the precipitated human proteins from the immune rabbit IgG. The banding pattern obtained with the protein extract used in the experiment is shown alongside for comparison. The molecular weight markers are shown to the right. Bands of 55 and 110 kDa are observed which are believed to correspond to the rabbit IgG γ chains. In addition, however, fainter bands at around 80 and 160 kDa are present which may represent monomeric and dimeric forms of the pulmonary protein to which the rabbit antibody was raised.

test). Immunohistochemical staining of A549 cell line cytospin preparations are shown in fig 2. IgG derived from plasma samples from patients with CFA, but not controls, gave strong positive cytoplasmic staining of the cells.

WESTERN BLOTTING WITH RABBIT IgG
Figure 3 illustrates the results obtained from blotting the control and immunised rabbit IgG on protein extracts from lung biopsy specimens

obtained from patients with CFA and A549 cells. Immune IgG, but not control IgG, recognised a protein in the 70–90 kDa region of blots produced with protein from both sources.

IMMUNOPRECIPITATION
Staining of polyacrylamide gels containing the precipitated human lung protein(s) and dissociated rabbit IgG revealed the presence of several distinct bands (fig 4). The most prominent band was identified at around 55 kDa with another at around 110 kDa. These appear to represent monomeric and dimeric

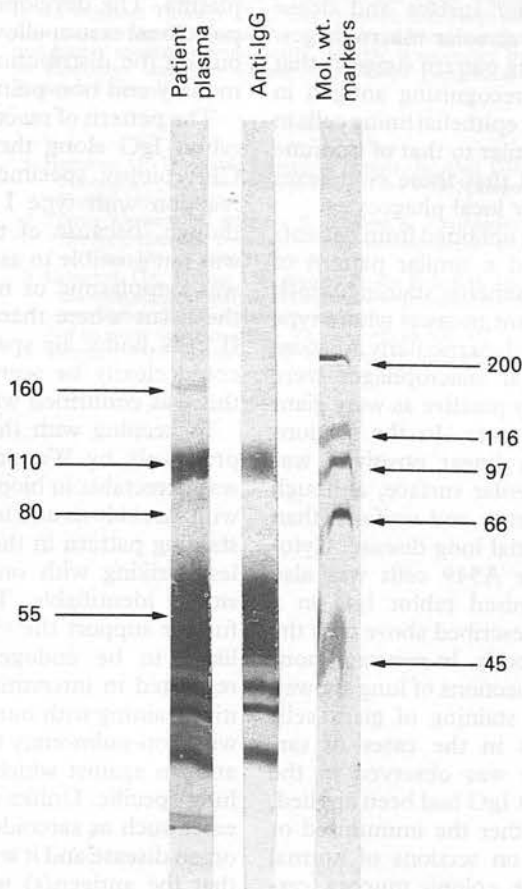


Figure 5 Western blotting of protein immunoprecipitated from lung biopsy material obtained from patients with CFA using immune rabbit IgG with and without patient plasma. The molecular weight markers are indicated on the right. The bands in the 80 and 160 kDa range are only detected in the presence of patient plasma indicating that they are not fragments of the rabbit IgG used in the experiment. The bands in the 50 and 110 kDa range, however, are positive in both blots confirming their nature as immunoglobulin.

IgG heavy chain (55 kDa) of the rabbit immunoglobulin used to precipitate the antigen. Further smaller bands were also noted at lower molecular weights and may represent either light chains or digestion fragments of the larger proteins.

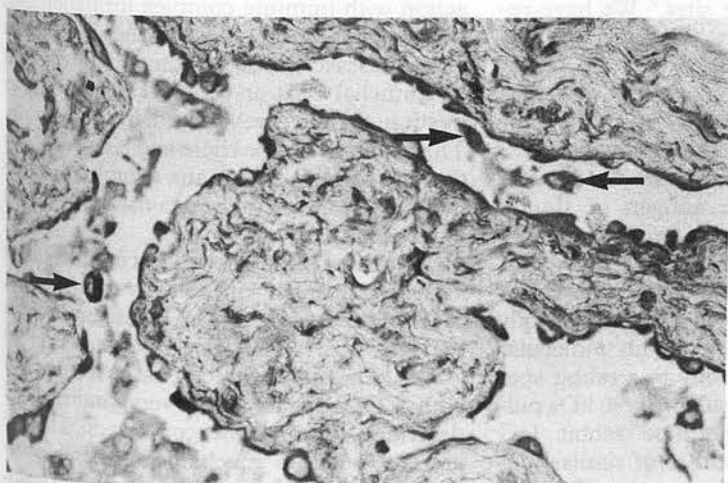


Figure 6 Cryostat section of an open lung biopsy specimen from a patient with CFA stained using immunised rabbit IgG and an immuno-alkaline phosphatase method with visualisation using the Vector Red substrate (original magnification $\times 250$). Positive staining of alveolar epithelial lining cells and some alveolar macrophages (arrows) can be identified.

In addition to bands attributable to the rabbit IgG, a band was identified in the 70–90 kDa region with a second around 150–160 kDa. The molecular weights of these bands correspond to the predicted position of monomeric and dimeric forms of the protein(s) which the rabbit and human autoantibodies recognise on Western blots performed with the lung protein extracts.

In order to confirm the nature of the various bands, the precipitated protein(s) were blotted in parallel, with and without patient plasma (fig 5). This was necessary as we were aware that the anti-human IgG antibody used in our detection system crossreacted with rabbit IgG. Using this strategy we were able to exclude the possibility that all the bands were due to rabbit immunoglobulin as the 70–90 and 150–160 kDa bands were only detected when the patient plasma was present. In addition, this confirmed that the 55 and 110 kDa bands were due to the rabbit immunoglobulin.

This result confirmed our results obtained by Western blotting which showed that the immunised rabbit IgG recognised a 70–90 kDa lung protein and that the protein to which the animal had raised antibodies was also recognised by the human CFA autoantibody.

IMMUNOHISTOCHEMISTRY

Sections from biopsy specimens obtained from patients with CFA stained with the immunised rabbit IgG (fig 6) showed strong positive staining along the edge of the alveolar surface in a manner suggestive of reaction with type I alveolar lining cells. In addition, strong cytoplasmic staining of hyperplastic type II cells was apparent and focal positivity was noted along the surface of ciliated bronchial mucosal cells. Alveolar macrophages were also noted to show cytoplasmic staining. This pattern of staining was very similar to that seen with the antibody to C1q (fig 7) which showed patchy

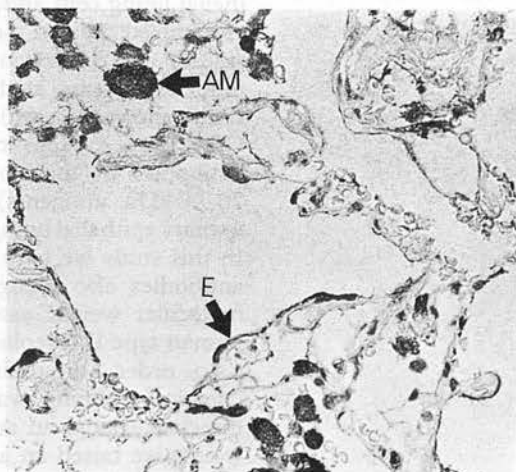


Figure 7 Formalin-fixed paraffin-embedded tissue from an open lung biopsy specimen obtained from a patient with CFA stained using an antibody to C1q in order to demonstrate the distribution of immune complexes in the lung (original magnification $\times 200$). Positive staining can be identified along the surface of the air space associated with the alveolar epithelial lining cells (E). In addition there is prominent cytoplasmic staining of alveolar macrophages present in the air spaces (AM).

positivity on the alveolar surface and dense cytoplasmic staining of alveolar macrophages. This similarity of staining pattern suggests that the rabbit antibody is recognising antigen in association with alveolar epithelial lining cells in a distribution that is similar to that of immune complex formation and that these complexes may be being cleared by local phagocytes.

Sections of lung tissue obtained from patients with sarcoidosis showed a similar pattern of reactivity with linear epithelial staining which appeared more prominent in areas where type II cells could be identified, particularly adjacent to granulomas. Alveolar macrophages were again noted to be focally positive as were giant cells within the granulomas. In the sections of control lung patchy linear positivity was observed along the alveolar surface, although this was much less intense and uniform than in patients with interstitial lung disease. Cytoplasmic staining of the A549 cells was also noted with the immunised rabbit IgG in a similar pattern to that described above with the native patient autoantibody. In contrast, non-immunised IgG on the sections of lung showed only faint cytoplasmic staining of giant cells within the granulomas in the cases of sarcoidosis. No positivity was observed in the sections where no rabbit IgG had been applied.

No positivity with either the immunised or control IgG was seen on sections of normal skin, tonsil, lymph node, colonic mucosa, cervix, liver, and kidney or cytopins of peripheral blood mononuclear cells, human T cell lines (H9 and Jurkat), and primary cultured chondrocytes. All were stained under identical conditions with the inclusion of A549 cell cytopins as a positive control in each experiment.

Discussion

CFA is believed to have an immunologically mediated pathogenesis. The nature and location of the antigen(s) are unknown but are believed to be associated with pulmonary epithelial lining cells and, perhaps, with capillary endothelial cells on the basis of ultrastructural studies⁶ and the previous demonstration of immune complexes at these sites.⁷ We have recently shown that patients with biopsy proven CFA have a high frequency of IgG autoantibodies in their plasma which recognise 70–90 kDa antigen(s) associated with pulmonary epithelial lining cells in tissue sections.⁵ In this study we have shown that these autoantibodies also recognise antigen of similar molecular weight associated with the A549 human type II alveolar cell line.

In order to confirm that the immunohistochemical staining was due to an antibody directed against an antigen of this molecular weight we raised an antibody in a rabbit specifically to the partially purified 70–90 kDa pulmonary antigen. The immune rabbit IgG obtained recognised protein(s) of similar molecular weight to the patient autoantibody in protein extracts produced from lung tissue from patients with CFA and A549 cells. In addition, the ~80 kDa protein precipitated by the rabbit IgG showed crossreactivity with the patient

plasma. The development of this anti-human polyclonal serum allowed studies to be carried out on the distribution of the antigen in pulmonary and non-pulmonary sites.

The pattern of reactivity with the immunised rabbit IgG along the alveolar surface of the CFA biopsy specimens was suggestive of a reaction with type I alveolar lining cells although, because of their flat morphology, it was not possible to assess whether the staining was cytoplasmic or membranous. In areas of the tissue where there were hyperplastic type II cells lining air spaces the staining pattern could clearly be seen to be cytoplasmic and this was confirmed with the A549 cells.

In keeping with the data we had obtained previously by Western blotting,⁵ the antigen was detectable in biopsy material from patients with sarcoidosis and in control lung tissue. The staining pattern in the control lung tissue was less striking with only patchy epithelial positivity identifiable. This result did, however, further support the view that the antigen was likely to be endogenous and may be up-regulated in interstitial lung disease. No positive staining with our rabbit IgG was obtained with non-pulmonary tissues suggesting that the antigen against which it was directed may be lung-specific. Unlike other interstitial lung diseases such as sarcoidosis, CFA is not a multi-organ disease and it would therefore seem likely that the antigen(s) which are responsible for the maintenance of the inflammatory process are restricted to the lung. Although the nature of the antigen is not yet known, pulmonary epithelial cells are undoubtedly highly specialised and it is therefore not surprising that they express unique antigens not present at other sites.

The significance of the autoantibody response in the pathogenesis of CFA is unclear. The antigen we have shown appears to have a cytoplasmic distribution, although we can detect it in bronchoalveolar lavage samples from some patients with CFA (unpublished data). This suggests that the antigen may be released from epithelial cells, possibly following cell injury. The resulting antibody–antigen interaction with immune complex formation could have a significant role in the perpetuation of the disease process, either by direct injury of epithelial cells or via local macrophage activation as they are cleared by phagocytosis. The results presented here and elsewhere⁷ show that the pattern of immune complex formation in CFA has a distribution consistent with such a hypothesis.

We believe that this study confirms that an antigen associated with alveolar epithelial lining cells is the target for IgG autoantibodies in patients with CFA. The distribution of this 70–90 kDa antigen in lung tissue from patients with CFA appears to be very similar to that described for immune complexes. This suggests a possible mechanism whereby autoantibodies combining with this antigen may, at least in part, account for some of the persisting immunological reaction observed in these patients, perhaps via complement activation and epithelial cell injury.

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Activation of peripheral blood mononuclear cells in bronchoalveolar lavage fluid from patients with sarcoidosis: visualisation of single cell activation products

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Abstract

Background – Interstitial lung diseases are characterised by the recruitment of mononuclear cells to disease sites where maturation occurs and activation products, including lysozyme (LZM), are released. Analysis of in vitro cell culture supernatants for activation products masks the functional heterogeneity of cell populations. It is therefore necessary to examine the secretion of activation products by single cells to assess whether the activation of newly recruited mononuclear phagocytes at the sites of disease in the lung is uniform and controlled by the local microenvironment.

Methods – The reverse haemolytic plaque assay was used to evaluate, at a single cell level, the ability of bronchoalveolar lavage (BAL) fluid from seven patients with sarcoidosis to activate Ficoll-Hypaque-separated peripheral blood mononuclear cells by comparison with BAL fluid from six normal volunteers and nine patients with systemic sclerosis. Monolayers of peripheral blood mononuclear cells and sheep red blood cells were cultured either alone or in the presence of 20% (v/v) BAL fluid with a polyclonal anti-LZM antibody. LZM/anti-LZM complexes bound to red blood cells surrounding the secreting cells were disclosed following complement lysis of red blood cells and quantification of plaque dimensions using microscopy and image analysis.

Results – Bronchoalveolar lavage fluid from all the patients with sarcoidosis increased LZM secretion by peripheral blood mononuclear cells compared with unstimulated mononuclear cells. By contrast, BAL fluid from the other individuals had no effect on LZM secretion.

Conclusions – Single cells activated by BAL fluid can be evaluated by the reverse haemolytic plaque assay. BAL fluid from patients with sarcoidosis, but not from patients with systemic sclerosis or normal individuals, contains components capable of activating mononuclear phagocytes to secrete lysozyme.

(Thorax 1994;49:1146-1151)

Sarcoidosis is characterised by the accumulation at disease sites of mononuclear cells

resulting in the formation of epithelioid cell granulomas.^{1,2} Circulating monocytes which are attracted to disease sites are subjected to the influence of local proinflammatory signals resulting in phenotypic and functional changes as they undergo maturation and activation.³ One of the activation products of stimulated mononuclear phagocytes is lysozyme (LZM),⁴ and measurements of LZM concentration have been used as an index of active disease.^{5,6} The primary function of LZM has always been considered bactericidal,^{4,7} even though previous work has shown that it may play a part in the modulation of monocyte-lymphocyte interactions⁸ and depression of neutrophil chemotaxis.⁹

If monocytes attracted to disease sites in sarcoidosis are stimulated to produce increased amounts of LZM, we hypothesised that factors within the local microenvironment are responsible for this change in function. To test the hypothesis we have used a method of identifying secretory products at the single cell level using the reverse haemolytic plaque assay to examine the effects of bronchoalveolar lavage (BAL) fluid on LZM secretion. The ability of the reverse haemolytic plaque assay to visualise cytokine products by single cells allows the modulation in function of cells to be assessed in the absence of cell-cell interaction and minimises paracrine effects of proinflammatory mediators from other cells in culture, thus enabling the direct effect of added BAL fluid on cell activation to be analysed. We therefore examined BAL fluid from patients with sarcoidosis, patients with systemic sclerosis, and normal individuals and compared the capacity of each to activate "naïve" monocytes to synthesise and secrete LZM.

Methods

PATIENTS

Three groups were studied: (1) patients with sarcoidosis (n=7; M:F 4:3; age 30.2 (1.6) years), (2) patients with fibrosing alveolitis associated with systemic sclerosis (n=9; M:F 4:5; age 44.6 (1.7) years), and (3) normal volunteers (n=6; M:F 3:3; age 26.3 (0.46) years). Of the patient groups, two patients with sarcoidosis were current smokers and three with systemic sclerosis were receiving treatment (prednisolone).

A diagnosis of sarcoidosis was confirmed by the presence of non-caseating granulomas in

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TENASCIN IMMUNOREACTIVITY IN CRYPTOGENIC FIBROSING ALVEOLITIS

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SUMMARY

Tenascin is a hexameric extracellular matrix (ECM) glycoprotein which has been demonstrated to have a temporal relationship with active scar formation in adult tissues. We hypothesized that this ECM protein might therefore serve to identify areas of active scarring in lung biopsies from patients with cryptogenic fibrosing alveolitis (CFA). The distribution of tenascin was examined in open lung biopsies from ten patients with CFA, six patients with sarcoidosis, and six pulmonary resection specimens from patients with no evidence of interstitial lung disease, using an immunohistochemical technique. Immunoreactive tenascin was not identified in histologically normal control lung parenchyma and was only focally found around large aggregates of granulomas in sarcoidosis. In the CFA, tenascin production was demonstrated in minimally damaged alveolar walls and areas of active disease but not in end-stage scarred lung. There was considerable local heterogeneity of staining within cases, which did not appear to relate to the density of the local inflammatory infiltrate. Large plaques of tenascin were noted to be particularly associated with hyperplastic type II alveolar epithelial lining cells, which are recognized to produce fibrogenic cytokines. The examination of tenascin expression in open lung biopsies from patients with CFA may be useful in assessing fibrogenic activity and may thus provide additional prognostic information.

KEY WORDS—tenascin; cryptogenic; fibrosing alveolitis; sarcoidosis; open lung biopsy

INTRODUCTION

Tenascin is a large, 190–240 kD, extracellular matrix glycoprotein with a hexameric, multi-domain structure composed of disulphide-linked subunits.¹ In the embryo, it appears to show temporal expression in many developing tissues including breast, lung, tooth, and kidney, but it appears to be absent from most normal adult tissues.² Production has, however, been shown in the stroma of some neoplasms^{3,4} and is locally increased during tissue repair.⁵ In experimental skin wounds, tenascin can be detected in the basement membrane region of the regenerating epithelium and in the subjacent granulation tissue. Following completion of the scarring process, tenascin expression ceases in the tissue.⁵ Although the exact role of tenascin in the healing process is unknown, this temporal expression suggests that it might be

used to identify areas of active scar formation. This might be of interest in assessing disease activity for conditions where prognosis is related to organ damage as a result of progressive scarring.

One such group of diseases is the interstitial lung diseases (ILDs). ILD represents a heterogeneous group of pulmonary conditions which result in thickening and often scarring of alveolar walls, with a resultant decrease in lung compliance and gas transfer.⁶ The commonest form of ILD is cryptogenic fibrosing alveolitis (CFA), which is of unknown aetiology but is believed to have an immunologically mediated pathogenesis⁷ with a persistent inflammatory reaction to as yet unidentified antigen(s) associated with alveolar epithelial lining cells.^{8–10} The disease is characterized by a chronic inflammatory cell infiltrate in the interstitium of the lung, with resultant scar formation and progression to end-stage honeycomb lung.¹¹ Response to therapy is usually poor and the median survival from the time of diagnosis is only 5 years.¹²

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The diagnosis of CFA can only be made with certainty following open or thoracoscopic lung biopsy and histological examination.¹³ This procedure also provides prognostic information, as it has been demonstrated that the more active the inflammation and the less fibrosis present, the better the prognosis for the patient.¹² This assessment, however, provides no direct information on the activity of the scarring process, which is likely to be important in the rate of decline in pulmonary function and ultimate survival.

In this study we have for the first time examined the distribution of immunoreactive tenascin in lung biopsies from patients with CFA at different stages of development and contrasted it with non-ILD control lung tissue and with sarcoidosis, another form of ILD which is usually associated with less severe pulmonary scarring.

MATERIALS AND METHODS

Lung tissue

Lung biopsy material was obtained from the archives of the Pathology Department, University of Edinburgh from ten cases of CFA and six cases of sarcoidosis which were clinically and pathologically well characterized. All biopsies had been performed at the Regional Thoracic Unit, City Hospital, Edinburgh for the investigation of clinically diffuse interstitial lung disease. Control, non-ILD lung tissue was obtained from six pulmonary resection specimens which had been performed for the diagnosis or treatment of pulmonary neoplastic lesions. Material for this study was selected so as not to include tumour or immediately adjacent lung tissue.

All the material had been routinely processed by fixation in neutral buffered formalin followed by embedding in paraffin wax. Representative blocks were selected from each case to show a range of pathological appearances and disease stages.

Immunohistochemical localization of tenascin

Tenascin expression in the tissue was demonstrated using the well-characterized monoclonal antibody BC4, which has been shown to react with a common epitope expressed by all human tenascin isoforms.¹⁴ Sections of lung were treated with 0.1 per cent trypsin at 37°C for 15 min prior to incubation with the antibody. Detection was performed using a standard ABC immunoperoxidase

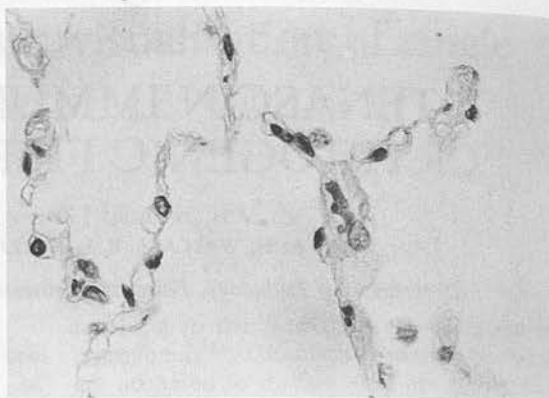


Fig. 1—Photomicrograph of a section of control, non-ILD lung tissue showing absence of immunoreactive tenascin in the alveolar septa (original magnification $\times 400$)

technique with visualization using diaminobenzidine.¹⁵ The sections were then counterstained with haematoxylin, dehydrated, and mounted.

RESULTS

Control lung tissue

The pulmonary parenchyma in the control lung tissue was largely negative for tenascin (Fig. 1). Focal positivity was noted, however, where mild non-specific thickening of alveolar walls could be identified. This was thought to be consistent with smoking-related changes in the lung and did not represent evidence of immunologically mediated ILD.

CFA

The distribution of immunoreactive tenascin in the biopsies was examined in relation to the patterns of disease that were apparent in the biopsies. These were defined as either (a) 'areas of active disease' where there was evidence of disruption in the tissue architecture with scarring of alveolar walls and a variable inflammatory infiltrate; (b) 'areas of end-stage disease' characterized by the presence of large dilated honeycomb spaces bounded by dense bands of collagen with minimal residual inflammatory activity; or (c) 'early disease' observed adjacent to areas of active disease, where the alveolar architecture was preserved but the alveolar walls were thickened and a mild interstitial inflammatory infiltrate was present.

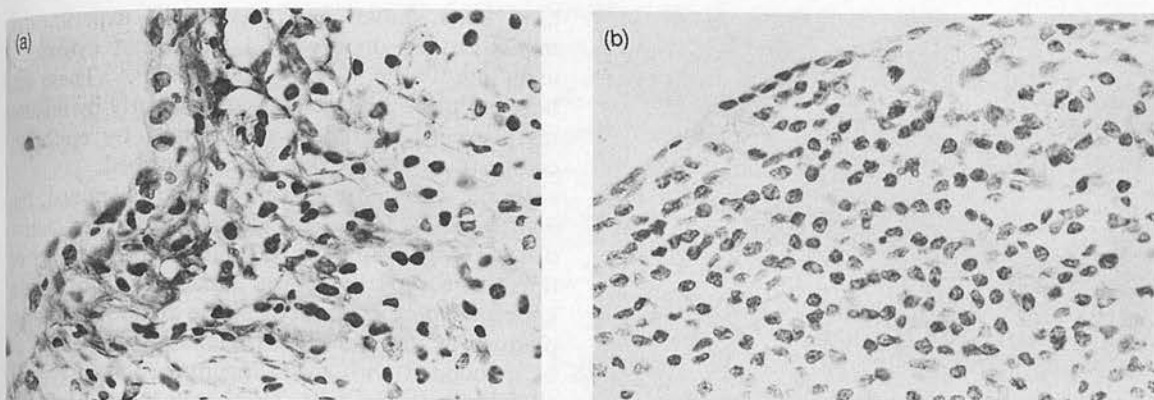


Fig. 2—(a) Photomicrograph of a section of lung tissue from a patient with CFA showing the distribution of immunoreactive tenascin in a subepithelial location associated with a local inflammatory cell infiltrate (original magnification $\times 400$). (b) Photomicrograph from the section illustrated in (a) demonstrating the absence of immunoreactive tenascin in an adjacent airspace despite the presence of a similar local inflammatory cell infiltrate (original magnification $\times 400$)

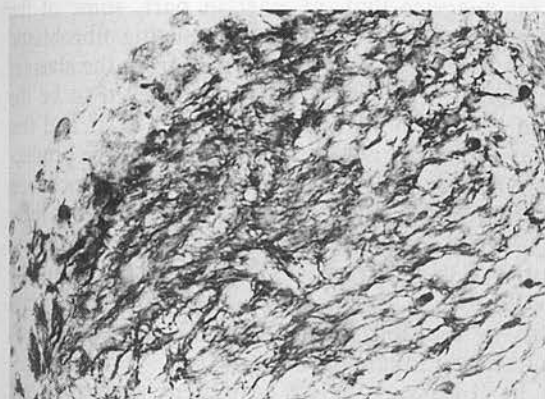


Fig. 3—Photomicrograph of a section of CFA lung tissue stained for the presence of immunoreactive tenascin. A large plaque of tenascin can be demonstrated in a subepithelial position which is associated with overlying hyperplastic type II alveolar epithelial lining cells (original magnification $\times 400$)

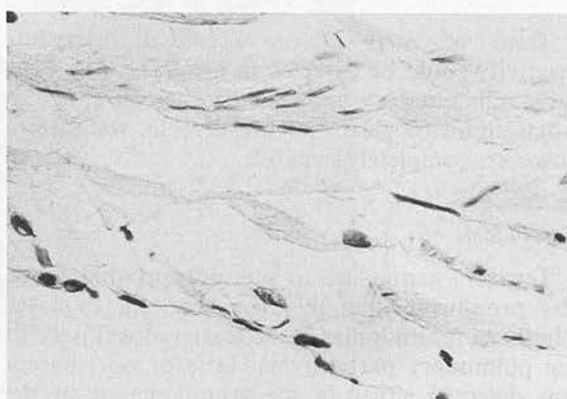


Fig. 4—Photomicrograph of an area of CFA lung tissue with end-stage scarring and honeycomb formation. No evidence of immunoreactive tenascin can be observed (original magnification $\times 400$)

Such areas were usually located in the subpleural region of the biopsy.

Areas of active disease—Tenascin reactivity was noted to be present immediately subjacent to alveolar epithelial lining cells. The distribution was, however, not uniform in any one airspace and there was considerable local variation in the intensity of staining (Fig. 2). Focally within the tissue, large plaque-like deposits of tenascin were observed beneath the basement membrane (Fig. 3) while immediately adjacent areas showed only scanty or no reactivity. These large plaques of tenascin appeared to be related to the presence of hyperplastic type II alveolar lining cells in the

airspaces, but not all airspaces lined by such cells showed this pattern of tenascin deposition. Tenascin immunoreactivity did not seem to relate to the density of locally infiltrating lymphocytes, macrophages, or other inflammatory cells, as both large plaques and scanty amounts were observed in areas of varying inflammatory activity. Foci of organizing intra-alveolar exudates were also identified and noted to be positive for tenascin.

Areas of end-stage disease—In cases where the biopsy revealed end-stage fibrotic honeycomb lung, no evidence of tenascin immunoreactivity could be identified (Fig. 4).



Fig. 5—Photomicrograph of alveolar septa in the subpleural region of a biopsy from a patient with CFA. Immunoreactive tenascin can be identified within the septa (original magnification $\times 400$)

Areas of early disease—Tenascin immunoreactivity could be detected in areas showing only early inflammatory and fibrotic changes (Fig. 5). Adjacent histologically normal alveolar walls were, however, completely negative.

Sarcoidosis

Tenascin expression in sarcoid appeared much less prominent than in CFA (Fig. 6). In cases where the granulomas were scattered diffusely in the pulmonary parenchyma, little or no tenascin was detected either in the granuloma or in the surrounding pulmonary parenchyma. However, in cases where the granulomas had become more confluent, patchy reactivity was noted to be present. This appeared to be most prominent between granulomas and did not extend out into the adjacent alveolar walls.

DISCUSSION

The results presented indicate that tenascin immunoreactivity in CFA has a sub-epithelial distribution, apparently limited to areas of the lung showing histological evidence of continuing disease activity. The biological control of tenascin expression in tissues is poorly understood. It is thought to be produced by fibroblasts in response to a variety of cytokines and growth factors¹⁶⁻¹⁸ and it is likely that the production of tenascin in CFA is regulated by similar mechanisms. Activation of fibroblasts and production of scar tissue

have been shown *in vitro* and in experimental models to be driven by a variety of cytokines, including TGF β , TNF α , and PDGF.¹⁹ These are believed to be produced predominantly by inflammatory cells, although production by epithelial cells in the lung¹⁹⁻²² has been described.

In the CFA cases that we have examined, the distribution of tenascin in the areas of active disease did not appear to relate to the number of inflammatory cells in the area. This divergence appeared most prominent in the areas where large plaques of immunoreactive tenascin appeared to be associated with overlying hyperplastic type II alveolar epithelial cells. These cells have previously been shown by immunohistochemistry and mRNA *in situ* hybridization to produce a variety of cytokines, including TGF β ,²¹ which has been shown to induce tenascin expression by fibroblasts *in vitro*.¹⁶ This suggests that, at least in part, some of the stimuli responsible for the persisting fibroblastic response in CFA may be derived from the alveolar epithelial cells. These cells are believed to be the site of immunological injury in CFA⁸⁻¹⁰ and this may lead to activation of the cells and the production of a variety of fibrogenic cytokines, including TGF β , TNF α , and PDGF.¹⁹⁻²² The role that these epithelial-derived factors may have on the disease process is at present unknown but potentially could form a significant source of these cytokines driving the fibroblastic process, independently of infiltrating inflammatory cells.

In ILDs such as CFA, patient morbidity and mortality are ultimately dictated by the rate of scarring that occurs in the patients' lungs. There is, however, no method of measuring the activity of the fibrotic process directly. Previous studies have approached this by analysing the local collagen subtypes in biopsy tissue²³ or by measuring pro-collagen peptides in bronchoalveolar lavage.²⁴ Tenascin is detectable in pulmonary parenchyma showing the earliest degree of scarring and is absent in end-stage disease, suggesting that demonstration of tenascin in CFA lung biopsy tissue might provide a more sensitive and specific mechanism of grading active fibroplasia in the tissue at the time of diagnosis than collagen. This idea is supported by its relative paucity in control lung tissue and in sarcoidosis.

The relationship of tenascin deposition to air-space epithelium and the knowledge that these cells, as well as immune cells, can produce fibrogenic factors suggest that a direct measure of active scarring independent of assessment of

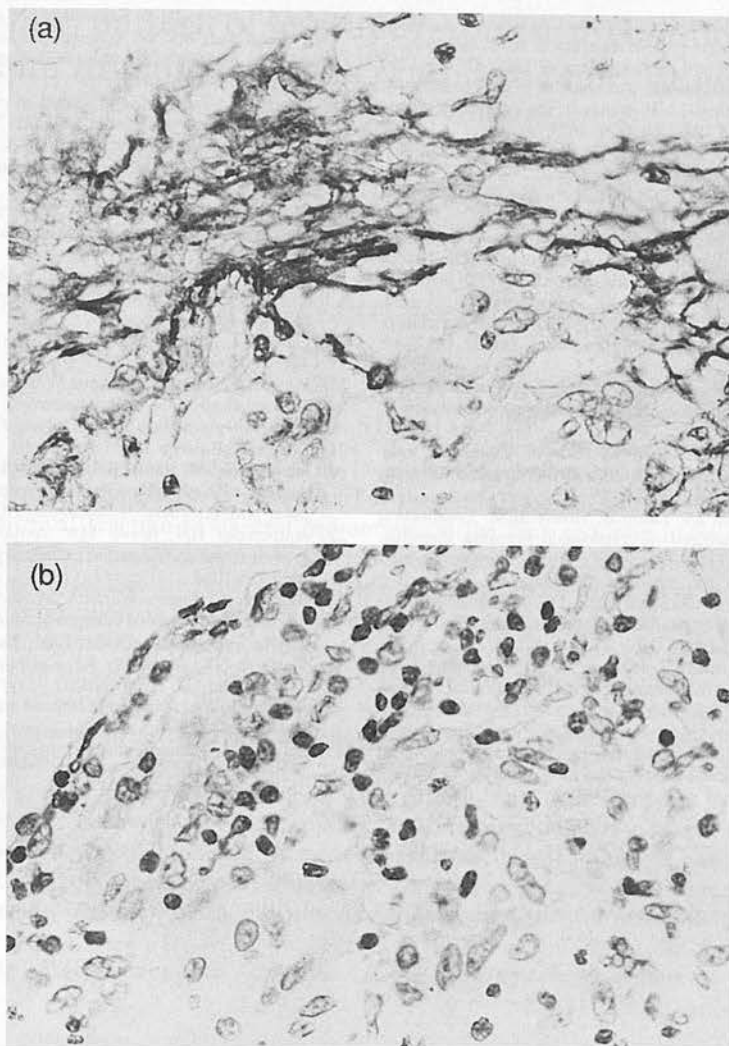


Fig. 6—(a) Photomicrograph from a lung biopsy obtained from a patient with pulmonary sarcoidosis showing the presence of immunoreactive tenascin around the periphery of a granuloma which was forming part of a larger confluent lesion (original magnification $\times 400$). (b) Photomicrograph of an isolated intra-pulmonary granuloma from a case of sarcoidosis showing the absence of immunoreactive tenascin around the lesion (original magnification $\times 400$)

immunological activity might be useful in predicting prognosis. The data presented here represent the first study of the pulmonary distribution of tenascin in patients with CFA at different stages of the disease. The temporal nature of its tissue expression, its apparent specificity to actively scarring lesions, and its paucity in normal adult lung tissue suggest that local tenascin expression or peripheral blood concentration²⁵ may provide such a marker.

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A type 2 (Th2-like) pattern of immune response predominates in the pulmonary interstitium of patients with cryptogenic fibrosing alveolitis (CFA)

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SUMMARY

CFA is an inflammatory condition of the lungs resulting in scarring, pulmonary failure and death. The etiology of the disease is unknown, but the pathogenesis is believed to involve a persistent immunological reaction to unidentified antigen in the lung resulting in tissue damage. Recent advances in our understanding of the immune system have shown that different patterns of stimulatory cytokines are produced at sites of inflammation by a range of cell types. Patterns of cytokine production by inflammatory cells are recognized to be associated with different patterns of immunological response, and these have been described as type 1 (or Th1-like) and type 2 (or Th2-like) on this basis. We have studied cytokine expression in the intestinal inflammatory cell infiltrate in lung tissue from patients with CFA using mRNA *in situ* hybridization and immunohistochemistry. Our results show that while there is evidence for both a type 1 (characterized by interferon-gamma (IFN- γ)) and type 2 (characterized by IL-4 and IL-5) response present in CFA, the type 2 (or Th2) pattern of cytokines appears to predominate. This would be consistent with a possible role for the humoral immune response in the pathogenesis of this condition. In addition, recent evidence suggests that IL-4 and IFN- γ may be important regulatory factors for pulmonary fibroblasts. The relative paucity of IFN- γ may contribute to the excessive fibroblast activation, deposition of collagen and scar formation that occurs in CFA.

Keywords fibrosing alveolitis cytokine IL-4 IL-5 interferon-gamma

INTRODUCTION

CFA is the commonest form of a heterogeneous group of pulmonary disorders known as the interstitial lung diseases (ILD) which are characterized by inflammation and scarring of the lung [1]. The etiology of CFA is unknown, but the pathogenesis is believed to involve a persisting immunological reaction to as yet unidentified antigen in the lung. Examination of affected pulmonary tissue shows the presence of a diffuse inflammatory infiltrate principally composed of T lymphocytes and macrophages with variable numbers of mast cells, neutrophils, eosinophils [2,3] and distinct B lymphocyte aggregates [4,5].

In order to understand the processes regulating inflammation and fibrosis in the lungs of patients with CFA it is necessary to try to dissect the network of cytokines that are produced by the infiltrating cells of the immune system as well as the resident structural cells. The role of individual cytokines is difficult to study, as the net effects on the various local tissue cells will be the result of a balance between different signals [6].

This makes the identification of patterns of cytokine production in diseases with an immunological pathogenesis important, as it may shed light on disease mechanisms.

Recent advances in our understanding of the immune response have identified subsets of CD4⁺ T helper cells (Th) by their ability to release different stimulatory cytokines, which can be shown to be associated with different patterns of immunological reaction [7-9]. A DTH reaction, or granulomatous pattern of response is associated with Th1 cells producing IL-2, IL-12 and interferon-gamma (IFN- γ), while those producing IL-4, IL-5, IL-6 and IL-10 are associated with an antibody-mediated response (Th2) and have been implicated in 'allergic' conditions such as asthma [10]. This situation is, however, complicated by the knowledge that such cytokines may, under some circumstances, also be produced by mast cells [11], monocyte/macrophages and CD8⁺ T lymphocytes [12].

The critical factor in determining the pattern and direction of an inflammatory response is, however, not necessarily dictated by the actual source of the different cytokines. It has, therefore, recently been suggested that the term 'type 1 response' be adopted when the cytokine profile is predominantly IL-2, IL-12 and IFN- γ and 'type 2 response' when the

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profile is predominantly IL-4, IL-5, IL-6 and IL-10, as this does not make the assumption that T helper cells are the only source of these factors [9].

Previous studies have suggested that most patients with CFA do not have elevated plasma levels of IFN- γ [13], suggesting that a type 2 pattern might predominate. In order to confirm this hypothesis it is necessary, however, to study the pattern of cytokine production by immunologically active cells within the interstitium of the lung, something that can only be achieved using human biopsy material. In order to determine if type 1 or type 2 pattern of cytokine production predominates in CFA, we examined open lung biopsy material for the presence of both mRNA and protein using a panel of reagents (IL-4, IL-5 and IFN- γ) selected to differentiate between the two patterns of response.

MATERIALS AND METHODS

CFA lung biopsy material

Ten open lung biopsies from patients with CFA were used (median age 70 years, range 61–78 years). All the biopsies were at least 2 cm in minimum dimension, most being performed using video-assisted thoracoscopy in the Regional Thoracic Surgical Unit, City Hospital, Edinburgh [14]. The histological features of all the cases were those previously described for CFA [2,3] and the clinical features were consistent with the pathological findings.

Trans-pleural injection of fixative was performed within 5 min of the biopsy being taken to ensure rapid fixation of the tissue and to improve histological appreciation of the lung architecture. All the tissue had been processed routinely in paraffin wax following fixation in 10% buffered formal saline. One block from each case was selected, and serial 3- μ m sections were cut onto Tespa-coated slides for mRNA *in situ* hybridization and immunohistochemistry as we have previously described [15].

Immunohistochemistry

Parallel sections from each case were stained with MoAbs to IL-4, IL-5 (both Genzyme, West Malling, UK) and IFN- γ (Ameri-

can Tissue Culture Collection Hybridoma no. HB 8291, deposited by Amgen Inc., Thousand Oaks, CA). These antibodies had been found to be suitable in our laboratory for use on formalin-fixed, paraffin-embedded tissue using the methods of microwave antigen retrieval [16] or with trypsin digestion. Immunohistochemical staining was performed using a standard avidin biotin complex-alkaline phosphatase or horseradish peroxidase (HRP) conjugate (both Dako, High Wycombe, UK) [17]. Visualization was performed using either the Vector red substrate (Vector Labs., Peterborough, UK) for alkaline phosphatase, or diaminobenzidine (Sigma, Poole, UK) for the HRP. The sections were counterstained with haematoxylin, dehydrated and mounted.

The percentage of diffusely infiltrating cells within the interstitium in each case staining positively with the MoAbs was quantified using the HOME microscope [18], an interactive image analysis system (Zeiss, Jena, Germany). For each case random high power fields ($\times 200$) were selected and identified on each of the sections stained with the different MoAbs. A minimum of 500 cells or three high power fields ($\times 200$) were then scored as either positive or negative for each antibody in each case.

mRNA *in situ* hybridization

In situ hybridization for IL-4, IL-5 and IFN- γ mRNA was performed on further parallel sections from each case using a non-isotopic method which we have previously described [15]. Briefly, the technique employed used exon cocktail FITC-conjugated probes (R+D Systems, Abingdon, UK) which were hybridized with the tissue sections overnight at 37°C following incubation of the tissue with proteinase K (Sigma).

Detection of the probe was carried out using a polyclonal rabbit anti-FITC antibody (Dako) which had previously been absorbed with a finely cut portion of normal human lung tissue to remove non-specific anti-lung activity before being recovered by affinity purification with Protein G (Pharmacia, Uppsala, Sweden). Sections were incubated with this antibody, washed and then further incubated with biotinylated swine anti-rabbit immunoglobulins (Dako). Visualization was performed using a streptavidin alkaline phosphatase system with the 5-bromo-4-chloro 3 indolyl phosphate (BCIP)/nitro blue tetrazolium

Table 1. Results of immunohistochemical staining for IL-4, IL-5 and IFN- γ on 10 cases of CFA

Patient	Age (years)	Sex	Per cent diffusely infiltrating mononuclear cells staining with monoclonal antibodies to each cytokine			
			IL-4	IL-5	IFN- γ	Ratio IL-4:IFN- γ
1	75	F	67.2	67.1	6.1	11.0
2	69	M	72.9	78.9	3.9	18.7
3	67	M	52.7	55.0	2.9	18.2
4	78	F	78.9	72.8	5.4	14.6
5	68	M	85.5	83.0	6.6	12.9
6	70	F	77.2	73.0	4.1	18.8
7	61	M	77.3	78.9	6.3	12.3
8	63	M	79.4	79.3	5.8	13.7
9	74	M	83.7	77.5	4.8	17.4
10	70	M	78.7	73.5	6.1	12.9
Median (range)			78.0 (52.7–85.5)	75.5 (55.0–83.0)	5.6 (2.9–6.6)	14.2 (11.0–18.8)

(NBT) substrate (both Sigma). This resulted in the production of a blue/purple reaction product at sites of hybridization between the probe and mRNA in the tissue section. No counterstain was used and the sections were mounted.

Controls, included in each experiment, were processed as above with the omission of the probe before hybridization or predigestion with mRNase.

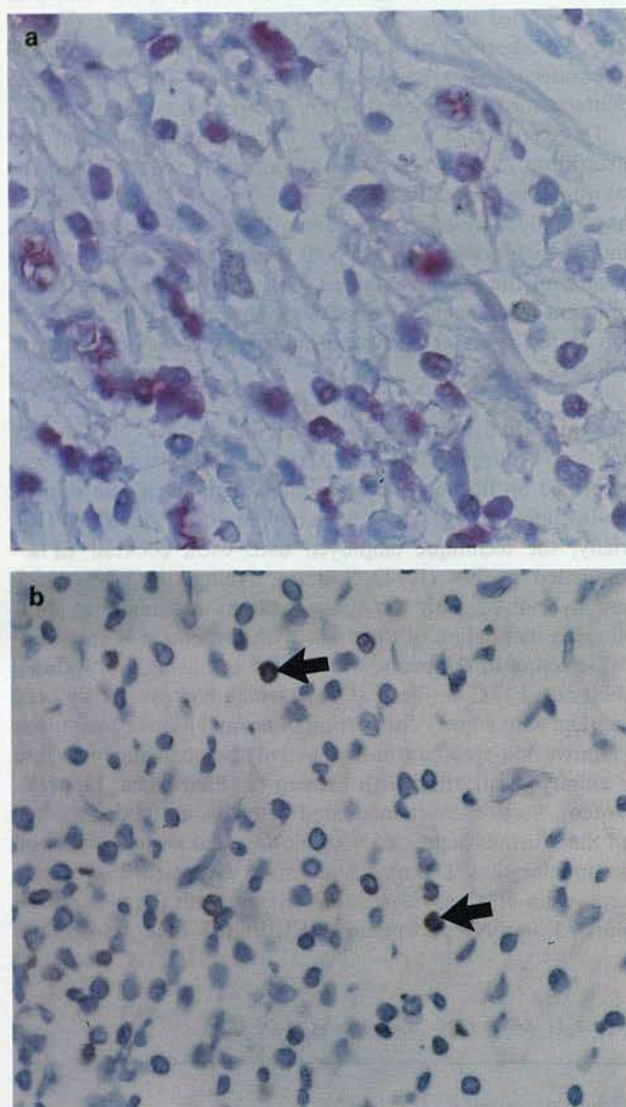
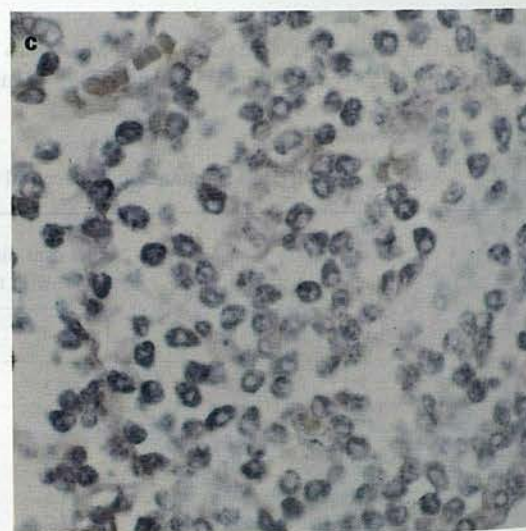
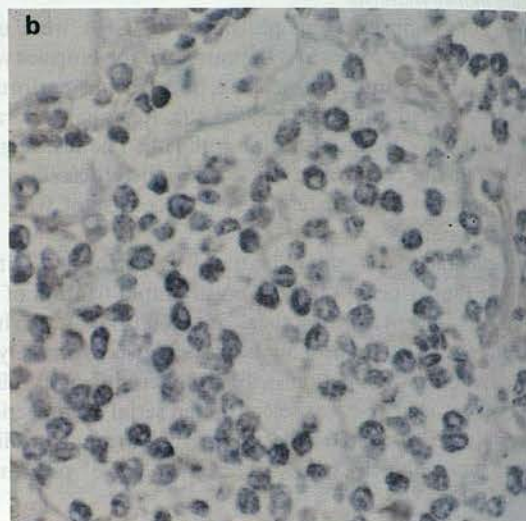
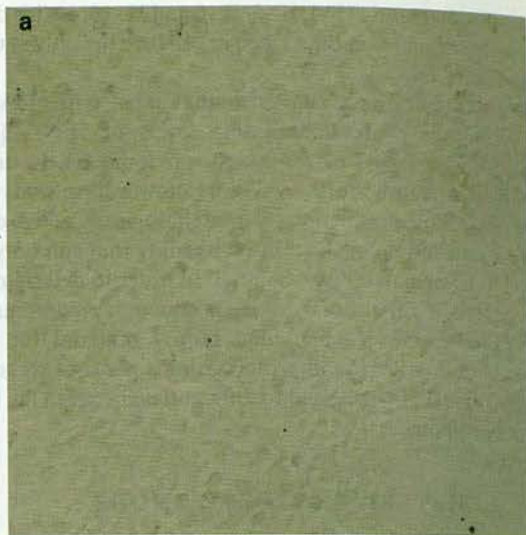


Fig. 1. Photomicrograph of CFA lung biopsy tissue stained with a MoAb antibody to (a) IL-4 (immuno-alkaline phosphatase method); and (b) IFN- γ (immunoperoxidase method). Over 50% of the diffusely infiltrating cells show positive red staining indicating the presence of immunoreactive IL-4. In contrast, only very few occasional infiltrating cells show evidence of positive staining (arrow) for immunoreactive IFN- γ (original magnification $\times 400$).

Fig. 2. Illustration of the results obtained using *in situ* hybridization. (a) Photomicrograph showing the appearance of a negative control which has been pretreated with mRNase, indicating the absence of any specific staining and confirming the specificity of the probes for RNA (original magnification $\times 200$). (b) Photomicrograph of CFA lung tissue probed for the presence of IL-4 mRNA (original magnification $\times 400$). Most cells in the tissue show cytoplasmic staining with nuclear clearing,



indicating the presence of IL-4 mRNA in the cells. (c) Photomicrograph of CFA lung biopsy tissue probed for the presence of IFN- γ mRNA (original magnification $\times 400$). Most cells in the tissue show cytoplasmic staining with nuclear clearing, indicating the presence of IFN- γ mRNA in the cells. The proportion of cells showing positivity for the mRNA contrasts with that in which the cytokine was detectable (Fig. 1b).

RESULTS

Diffusely infiltrating inflammatory cells

The results of the immunohistochemical studies are shown for each individual case in Table 1. Most of the diffusely infiltrating mononuclear cells stained positively for IL-4 (Fig. 1a) and IL-5 (>90%) in all of the cases. In contrast only a small minority (<7%) of the cells stained for IFN- γ (Fig. 1b). No apparent difference in the relative proportion of cells positive for any of the three cytokines was evident in the cases examined, nor was there evidence of significant variation in the ratio of IL-4 to IFN- γ -positive cells. These results suggest that a type 2 pattern of cytokine production predominated in the interstitium of patients with CFA.

Most of the infiltrating mononuclear cells within the interstitium of all 10 cases were identified as positive for IL-4 (Fig. 2a), IL-5 and IFN- γ (Fig. 2b) mRNA. In contrast to the results obtained immunohistochemically, the proportion of cells that were positive with each probe appeared similar when assessed qualitatively. This suggested that the majority of cells in which IL-4 and IL-5 were detected were likely to be synthesizing it, and that IFN- γ mRNA was being transcribed in cells that were not producing the cytokine.

Lymphoid aggregates

In six of the cases discrete 'B' lymphoid aggregates were identified. Most of these cells stained immunohistochemically with the antibodies for IL-4 (Fig. 3a) and IL-5, but only a few IFN- γ -positive cells were identified. In contrast with the results obtained for the diffusely infiltrating cells, only a very few cells within the aggregates could be identified which showed evidence of IL-4 (Fig. 3b), IL-5 or IFN- γ mRNA. This suggested that the immunohistochemical staining of the majority of the cells in the aggregates may be due to IL-4 and IL-5 associating with the cells, rather than the result of synthesis.

DISCUSSION

We postulated that CFA might be associated with a type 2 (Th2 like) response, as IFN- γ levels are recognized to be normal in most patients with CFA, unlike those with sarcoidosis [13]. In addition, other workers have shown evidence of humoral immune activity in the lung [19–22], and we have recently described the presence of IgG autoantibodies to an alveolar epithelial antigen [23,24], all of which would also support the concept of a type 2 pattern of cytokine production. The results presented here support this hypothesis, as only a small percentage (<5%) of the infiltrating cells showed positivity for the key type 1 cytokine IFN- γ , while the majority (>50%) were positive for the type 2 cytokines IL-4 and IL-5.

We used both mRNA *in situ* hybridization and immunohistochemical identification of the cytokine in this study, as this combination affords the most logical method to study cytokine production in tissue sections. mRNA *in situ* hybridization clearly identifies cells capable of synthesizing the cytokine rather than those which may have it bound to cellular receptors, while the immunohistochemistry confirms that translation of the mRNA has occurred. This second step is essential, as it is recognized that expression of cytokine mRNA may not correlate well with actual production of the protein [11].

Discrepancy between the frequency with which IFN- γ

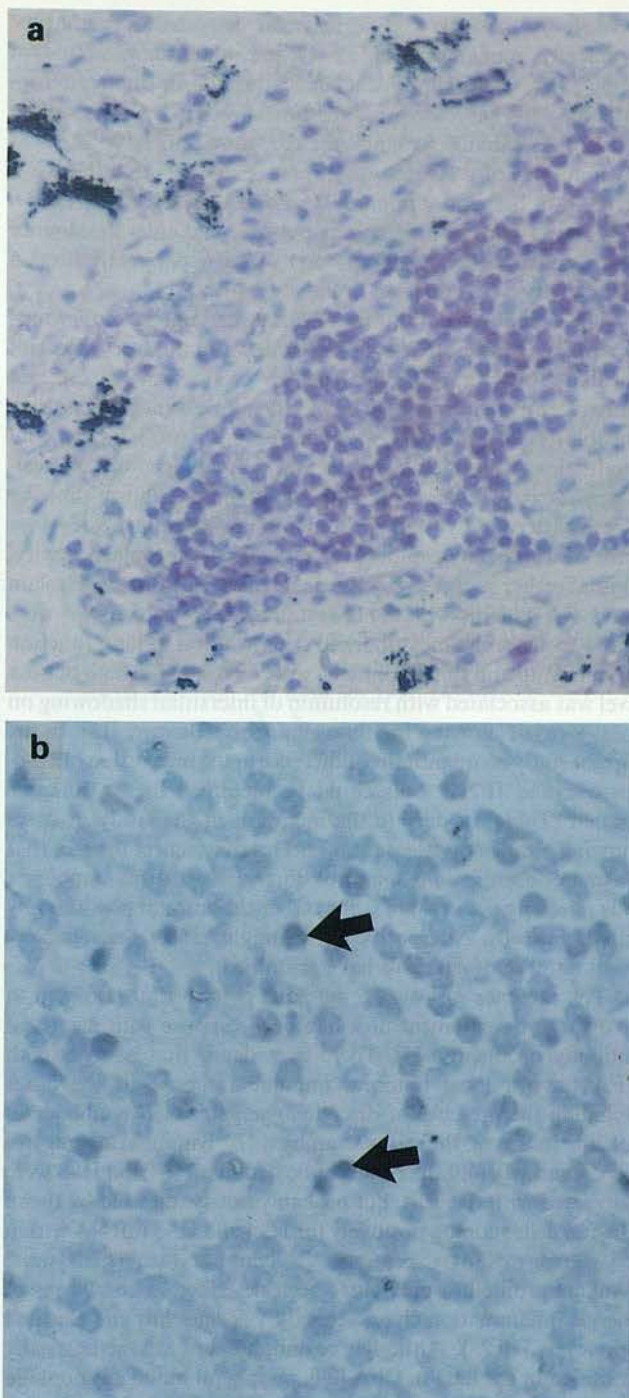


Fig. 3. Photomicrograph of a B lymphoid aggregate in CFA lung biopsy tissue stained with (a) a monoclonal antibody to IL-4 (immuno-alkaline phosphatase method; original magnification $\times 100$); and (b) probed for the presence of IL-4 mRNA (original magnification $\times 400$). The results indicated that most of the cells in the aggregate show evidence of immunoreactive IL-4 but that the mRNA can be detected in only occasional cells within the aggregate (arrow).

mRNA and cytokine was detected suggests that following activation, cells transcribe multiple gene sequences but that there is subsequent tighter regulation of translation of the mRNA to the protein product. An alternative explanation, that the cytokine after translation from the mRNA was rapidly

secreted by the cells and so was not detected immunohistochemically, appears unlikely as we were able to detect abundant cellular IFN- γ immunohistochemically in tissue obtained from patients with sarcoidosis (data not shown). Unlike patients with CFA, sarcoid patients are recognized to have elevated plasma levels of the cytokine [13]. This suggests that there is a correlation between immunohistochemically detectable IFN- γ in the lung and its presence in plasma, and thus its absence above normal levels in the plasma of CFA patients reflects a true paucity of production in the lung.

The significance of this in CFA is uncertain. Several groups of workers have noted that IFN- γ has an inhibitory effect of fibroblast proliferation and collagen production [25,26], and more recently Sempowski *et al.* has identified a subset of murine fibroblasts for which IL-4 promotes both proliferation and production of both collagenous and non-collagenous extracellular matrix proteins [27]. This raises the possibility that the type 2 pattern of cytokine production may favour the development of scarring by an imbalanced action on fibroblast activity. This is further suggested by the observations of Prior & Haslam that CFA patients with the lowest plasma levels of IFN- γ were found to show the most marked deterioration in lung function on follow up and that in patients with sarcoidosis a high plasma level was associated with resolution of interstitial shadowing on chest x-rays following immunosuppressive therapy [13]. In the current study no quantitative difference in the proportion of IL-4 positive and IFN- γ cells could be identified in the 10 cases studied (Table 1), despite the inclusion in the study of cases showing a range of disease stages. This may reflect the fact that plasma concentrations may not correlate with the number of cells releasing the cytokine, but rather the amount produced by individual cells. Alternatively, this might reflect the relatively small number of cases we have examined.

The presence of a type 2 cytokine pattern is also known to favour the development of a humoral response with persistent antibody production [7]. There is evidence that patients with CFA have a local humoral immune response in the lungs including the presence of discrete aggregates of B lymphocytes [19,20]. In six of the cases examined 'B' lymphoid aggregates were identified, and the results indicated that IL-4 and IL-5 were associated with the cells, but probably not synthesized by them. The few cells staining positively for IL-4 and IL-5 mRNA within the aggregates may represent T lymphocytes. The type 2 cytokine profile has previously been described in conditions of allergic inflammation characterized by eosinophils and elevated IgE levels [10,28]. Although eosinophils are characteristically identified in the lung in CFA [29], peripheral blood eosinophilia and elevated IgE levels are not observed [30]. The reasons for this are unclear, but *in vitro* studies have indicated that IL-8 can selectively inhibit IgE production by IL-4-stimulated B lymphocytes [31]. IL-8 is recognized to have an important role in the pathogenesis of the alveolitis observed in CFA [32]. This suggests that CFA may represent a modulated type 2 response with persistent antibody production in the absence of allergic features.

We conclude from this study that CFA patients show a type 2 (Th2-like) pattern of cytokine production by the infiltrating interstitial inflammatory cells. The significance of this in terms of the pathogenesis of the disease is at present unknown, but is consistent with a role for the humoral immune response. This further supports the concept that in CFA the pattern of immune activation results in a relative imbalance of factors

which regulate fibroblast function, and may contribute to the subsequent progressive scarring which characterizes this condition.

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